Neurogenic Lower Urinary Tract Dysfunction in Spinal Cord Injured Rats:

Establishment of a New Translational Urodynamic Model, Analysis of Lower Urinary Tract Regulating Neuronal Circuits and Treatment Effects of anti-Nogo-A Antibody

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1.1 Zusammenfassung

Die physiologische Blasenfunktion ändert sich dramatisch nach Rückenmarksverletzungen. Fast alle Patienten leiden an neurogener Blasenfunktionsstörung. Am gefährlichsten ist die Detrusor Sphinkter Dyssynergie, hierbei kontrahiert der Schliessmuskel simultan zum Blasenmuskel (dyssynerge Kontraktion) und die Blasenentleerung wird massiv gestört. Das führt zu unzureichender Entleerung mit sehr hohem Blasendruck und grossem Restharn Volumen. In der Folge kommt es zu Infektionen der Harnwege und Nieren Reflux. Diese Konstellation ist lebensbedrohlich: wenn nicht angemessen behandelt, verursacht sie Nierenversagen. Es gibt nur symptomatische Behandlungen, die aktuelle Gold-Standard-Therapie ist die vier- bis sechsmal tägliche Selbst-Katheterisierung. Alles zusammen führt zu enormen sozialen und wirtschaftlichen Kosten für die rückenmarksverletzten Patienten, ihre Familien und die Gesellschaft. Deshalb zählt die Blasen- und Darmfunktion auch heute zu den höchsten Prioritäten in der Rehabilitation. Erste kausale Behandlungsmöglichkeiten sind dringend erforderlich. In den späten 1990er Jahren haben Schwab und Kollegen einen Antikörper gegen ein zentrales Nervensystem Myelin Protein, Nogo-A, entwickelt. Die Blockierung von Nogo-A führt zu substanzieller axonaler Sprossung *in vitro* und funktioneller Erholung *in vivo*. Nogo-A destabilisiert das Zytoskelett über den rho / ROCK Signalweg, verursacht einen Kollaps von Wachstumskegeln, hemmt zusätzlich das Wachstum und die neuronale Plastizität durch Herunterregulierung von wachstumsassoziierten Genen. Liebscher und Kollegen haben im Jahr 2005 gezeigt, dass bei Anti-Nogo-A Antikörper behandelten, rückenmarksverletzten Tieren die selbständige Blasenentleerung signifikant schneller möglich war als in Kontroll-Antikörper behandelten Tieren. Daher war unsere Arbeitshypothese, dass die Anti-Nogo-A-Antikörper-Behandlung die erste Behandlungsoption der Ursache von neurogener Blasenfunktionsstörung durch Rückenmarksverletzung sein könnte. Initialer Teil der Arbeit war die Entwicklung eines ersten Urodynamik-Modells welches ermöglicht wiederholte Messungen an den vollkommen wachen Ratten durchzuführen,

einschliesslich der Beurteilung der äußeren Harnröhrenschließmuskelfunktion. Wir fanden signifikante Unterschiede im Vergleich zur derzeitigen Standard Methode mit Urethan narkotisierten Tieren. Das deutet auf eine echte Translationalität unseres neuen Modells hin. In einem zweiten Teil der Arbeit wurde die mögliche Wirkung von Entzündungsgewebe um die Blasenkatheter Implantationsstelle auf die Blasenfunktion fokussiert. Wir fanden keine Unterschiede in der Blasenfunktion zwischen naiven und Katheter implantierten Tieren. So ist es wahrscheinlich, dass unsere wach-urodynamischen Messungen wirklich die physiologische Funktion der Blase reflektieren. Wir haben nun diese Methode verwendet, um Ratten mit unvollständigen Rückenmarksverletzung auf der T8 Ebene zu untersuchen. Wir fanden massiv erhöhte Hochfrequenz Salven in der Elektromyographie (EMG) Aktivität des äußeren Harnröhrenschließmuskels, beginnend ab drei Wochen nach Rückenmarksverletzung. Das ist eine sehr vergleichbare Situation mit der menschlichen Detrusor Sphinkter Dyssynergie. Im dritten Teil dieser Arbeit haben wir uns auf die anti-Nogo-A Antikörper Behandlung konzentriert, als mögliche erste ursächliche Behandlungsoption für neurogene Blasenfunktionsstörung, insbesondere Detrusor Sphinkter Dyssynergie. Wir fanden in inkomplett rückenmarksverletzten Tieren behandelt mit Anti-Nogo-A Antikörper eine signifikante Abnahme. Das heißt eine physiologischere Situation in Schlüssel Urodynamik Parametern wie dem maximalen Blasendruck und der Blasenentleerungszeit im Vergleich zu Kontroll-Antikörper behandelten Tieren. Die Anti-Nogo-A Antikörper-Therapie hat bei Tieren mit inkompletter Rückenmarksverletzung wichtige Urodynamikwerte signifikant in Richtung der physiologischen Situation verändert, dies im Vergleich zu Kontroll-Antikörper behandelten Tieren. Die Anti-Nogo-A Antikörper-Therapie ist somit wahrscheinlich die erste kausale Behandlungsoption für die neurogene Blasenfunktionsstörung. Studien am Menschen haben nun als nächsten Schritt höchste Priorität.

1.2 Summary

Normal bladder function changes dramatically after spinal cord injury, where in almost all patients neurogenic lower urinary tract dysfunction appears and persists long term. Most dangerous is the detrusor sphincter dyssynergia: The sphincter muscle contracts during voiding (i.e. is dyssynergistic) and disturbs micturition. This results in insufficient voiding with large post void residual urine, causing urinary tract infections and very high intravesical pressure, leading to renal reflux. The combination is life threatening and, if not appropriately treated, causes kidney failure over time. There are currently only symptomatic treatments available. Enormous social and economic costs are the result for spinal cord injury patients, their families and society. Thus, bladder and bowel dysfunction are rated among the highest rehabilitation priorities by spinal cord injury patients, and causal treatment options are urgently needed. In the late 1990's, Schwab and colleagues developed an antibody against a central nervous system myelin protein named Nogo-A. Blocking of Nogo-A induced substantial axonal sprouting and functional recovery *in vitro* and *in vivo*. Nogo-A destabilizes the cytoskeleton via the rho/ROCK pathway causing growth cone collapse and inhibits neuronal growth and plasticity by down-regulation of growth-associated genes. Liebscher and colleagues have shown in 2005 that anti-Nogo-A antibody treated spinal cord injury animals recovered independent bladder voiding significantly faster than control antibody treated animals. Hence our working hypothesis was that anti-Nogo-A antibody treatment might be the first causal treatment option for neurogenic lower urinary tract dysfunction due to spinal cord injury. Part one of the thesis was the development of the first urodynamic model allowing for repeated measurements in the fully awake rats, including assessment of external urethral sphincter function. We found significant differences in comparison to current standard methods, using urethane anaesthetized animals, pointing out the importance of truly translational animal models. A second part of the thesis was focused on the potential effect of inflammatory tissue around the implantation site of the bladder catheter. No difference was found between naïve and catheter implanted animals for bladder parameters; thus, it is likely that awake

urodynamic measurements truly reflect physiological bladder function in rats. We then used this method to investigate rats with incomplete spinal cord injury at T8 level where we found massive increased high frequency bursting in electromyography (EMG) activity of the external urethral sphincter that appeared and remained three weeks after spinal cord injury during voiding. This represents a highly comparable situation to human detrusor sphincter dyssynergia. The third part of this thesis was focused on the investigation of anti-Nogo-A antibody treatment as a potential first causal treatment option for neurogenic lower urinary tract dysfunction, in particular detrusor sphincter dyssynergia. We found in incomplete spinal cord injury animals, treated with anti-Nogo-A antibody, a significant decrease, i.e. more physiological situation, in key urodynamic parameters such as maximal bladder pressure and voiding time compared to control antibody treated animals. Anti-Nogo-A antibody therapy has the potential to be the first causal treatment option for neurogenic lower urinary tract dysfunction and human trials are highly indicated as the next step.

1.3 Table of content

1.4 List of abbreviations

LUT	Lower urinary tract
LUTD	Lower urinary tract dysfunction
NLUTD	Neurogenic lower urinary tract dysfunction
DSD	Detrusor sphincter dyssynergia
UDI	Urodynamic investigation
Pves	Intravesical pressure
Pdet	Detrusor pressure
Pabd	Abdominal pressure
EMG	Electromyography
CNS	Central nervous system
CSF	Cerebrospinal fluid
CST	Corticospinal tract
SCI	Spinal cord injury
AB	Antibody

2 Introduction

2.1 The lower urinary tract and it`s neuronal control

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M.P.S.: Produced all the figures. Wrote the manuscript (edited by T.M.K.).

2.1.1 Abstract

The function of the lower urinary tract is simple: urine storage and voiding. However, despite this simplicity of function, the control of the lower urinary tract is a rather complex system involving both the peripheral (pelvic parasympathetic, lumbar sympathetic and somatic pudendal nerve) and central (spinal and supraspinal) nervous system. The lower urinary tract is comprised of the bladder, the urethral sphincter and the urethra and it is controlled by neural pathways from the spinal cord and brain via a complex set of peripheral somatic and autonomic nerve fibers. Following an initial learning process in childhood, bladder control is achieved allowing volitional voiding with voluntary relaxation of the external urethral sphincter when it is deemed appropriate. The high prevalence of lower urinary tract dysfunction in neurological diseases reflects the complexity of the control of the lower urinary tract in health. The site and nature of the neurological lesion determines the pattern of lower urinary tract dysfunction resulting in symptoms that have a pronounced effect on quality of life. Moreover, especially spinal lesions leading to detrusor sphincter dyssynergia (DSD) may jeopardize the upper urinary tract and need appropriate treatment to prevent renal failure.

2.1.2 Anatomical structures and functions of the lower urinary tract

The lower urinary tract comprises of two functional main structures: a reservoir, the urinary bladder, as well as the drainage and continence system, consisting of the urethra, bladder neck and sphincter (Figure 1) (Mangera et al., 2010). Males and females have several differences regarding pelvic anatomy and sexual function, most prominent is the supplementary prostate in males that may influence lower urinary tract function in particular with increasing age (Thorpe & Neal, 2003).

The urinary bladder (vesica urinaria), a hollow muscular organ, has two main functions (Mangera et al., 2010):

Low-pressure reservoir for the storage of urine (Figure 1b)

By the kidneys produced urine is guided through the ureters to the bladder. The normal adult bladder has a good compliance, i.e. accepts large volumes without markedly increasing pressure and has a total capacity of about 500mL.

To generate enough pressure for appropriate emptying without post void residual (Figure 1c)

When time and place are convenient, contraction of the detrusor and gain of intra-abdominal pressure lead to an increase of the intra-vesical pressure until the leak point pressure is reached and the intra-vesical pressure overshoots the sphincter pressure, hence voiding begins.

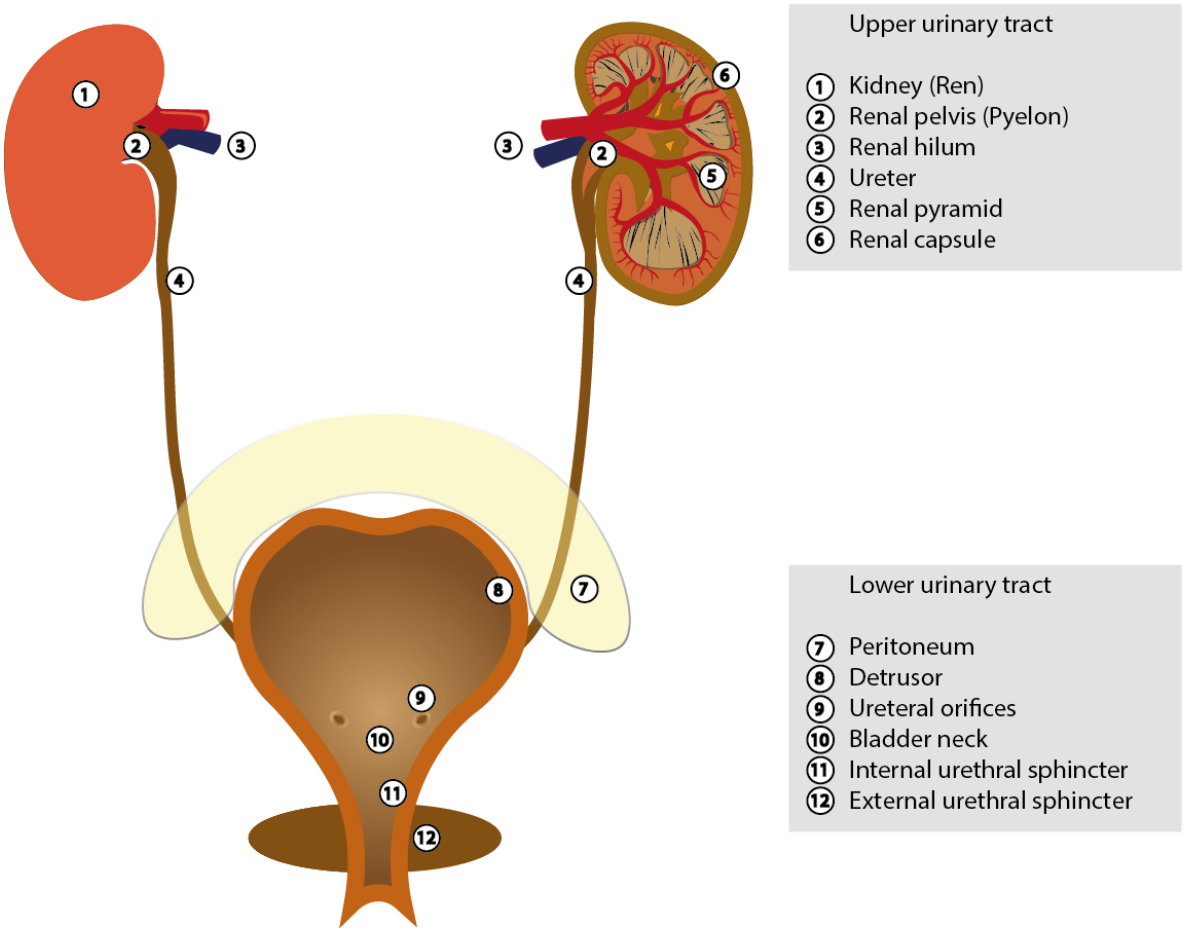
The position of the bladder varies extensively related to the filling stage. The empty bladder is place behind the symphysis pubis and lies exclusively extraperitoneal in the pelvis having the shape of a reverse pyramid-like structure. The complete filled bladder reaches with a balloon-like shape wide in to the abdominal cavity and may reach with the bladder dome in its rostral extension up to the umbilicus.

The detrusor consists of three directed layers of smooth muscle fibers (Dixon & Gosling, 1983). One layer of circular smooth muscles is sandwiched between two

longitudinal muscle layers (Gabella & Uvelius, 1990). The luminal part of both the bladder and urethra is covered by the urothelium, a layer of transitional epithelium which acts as linear and almost impermeable barrier allowing the bladder to store the urine (Lewis, 2000). Lose connective tissue, the lamina propria, is linking the urothelium with the detrusor and contains a plexus of afferent nerve fibers, capillaries, immune cells and lymph vessels (Inou et al., 1992). The urothelium together with the lamina propria is called mucosa.

Figure 1

a. Schematic view of the upper and lower urinary tract



b. Storage - sympathetic

c. Voiding - parasympathetic

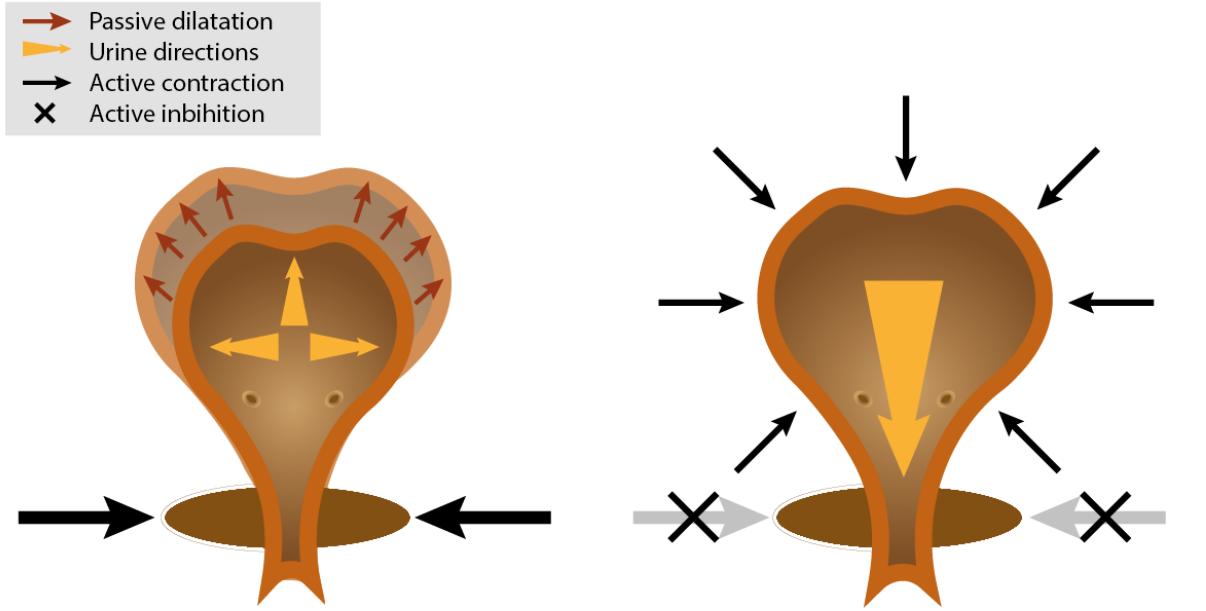


Figure 1: **a.** Schematic view of the upper and lower urinary tract; **b.** illustration of the sympathetic mediated storage phase; **c.** illustration of the parasympathetic mediated voiding phase.

The storage and voiding system, consisting of the bladder, (internal / external) urethral sphincter and urethra has two main functions:

Storage of urine (Figure 1b)

The bladder neck / internal urethral sphincter and the external urethra are contracted in the storage phase thereby increasing the infra-vesical resistance preventing leakage. The external urethral sphincter is under somatic control, allowing e.g. direct interruption of voiding.

Voiding (micturition) (Figure 1 c)

The bladder neck / internal urethral sphincter and the external urethral sphincter are relaxed during voiding allowing normal low pressure emptying of the bladder.

The lower part of the bladder is channeling in to the bladder neck and urethra. The bladder neck consists of oriented smooth muscles in three different layers (outer – longitudinal, middle – circumferential, inner – longitudinal) (Mangera et al., 2010). The middle – circumferential smooth muscle layer proceeds along the urethra and is called internal urethral sphincter. Where the urethra penetrates the pelvic floor muscles, encases the horseshoe shaped external urethral sphincter muscle the urethra. The external urethral sphincter is composed of circular voluntary controlled striated muscles, that are part of the pelvic floor muscles.

2.1.3 Peripheral innervation of the lower urinary tract

The lower urinary tract is innervated by three different pathways of the nervous system (Figure 2/3) (Fowler et al., 2008) :

Sympathetic fibers that travel from the thoracolumbar spinal cord along the splanchnic and hypogastric nerve

Parasympathetic fibers that pass from the sacral spinal cord along the pelvic plexus

Somatic motor fibers that emerge at the sacral spinal cord and lay in the pudendal nerve

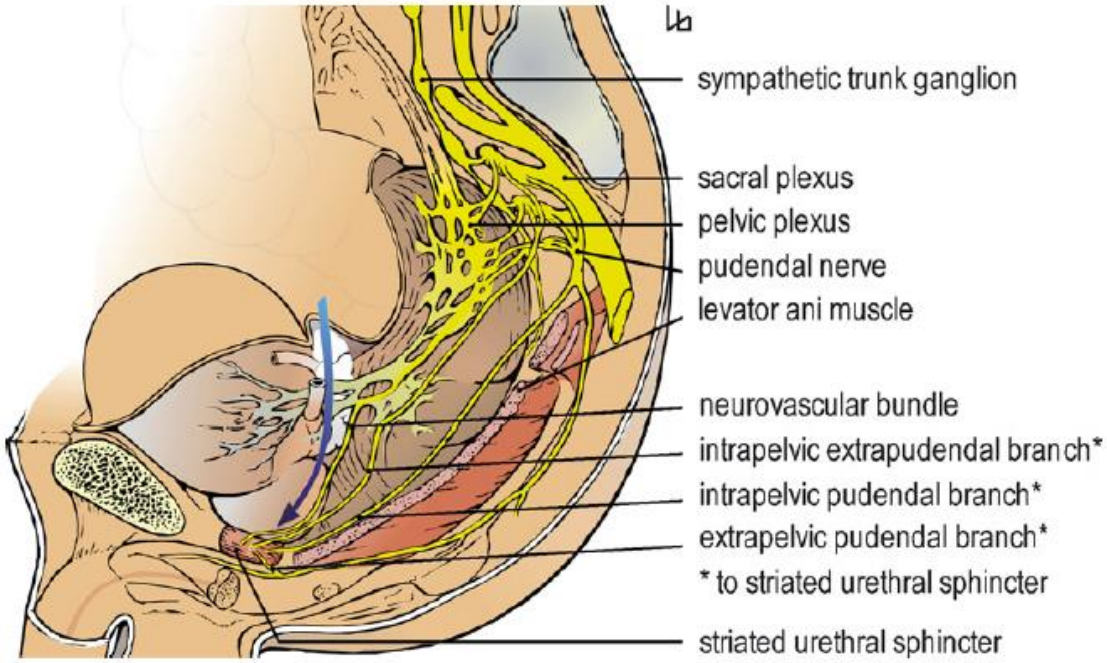


Figure 2: Schematic view of the innervation of the lower urinary tract. From (Kessler et al., 2005)

Both the sympathetic and the parasympathetic pathways are switched through a ganglion, hence consist of pre- and post-ganglionic (or synaptic) fibers. Sympathetic fibers are trans-synaptic linked either in the sympathetic chain ganglia or the inferior mesenteric ganglia, whereas parasympathetic signals are switched in the major pelvic ganglion from pre- to post-synaptic fibers. Somatic fibers innervate from the spinal cord the external urethral sphincter directly without trans-synaptic switch. All three nerve pathways of the lower urinary tract, namely the hypogastric nerve, pelvic plexus and pudendal nerve, carry afferent fibers of the corresponding innervation regions back to the spinal cord. Afferent signals are of outstanding importance to orchestrate the sympathetic mediated storage and the parasympathetic mediated voiding phase.

Sympathetic pathways: Sympathetic preganglionic neurons are located in the intermediolateral nucleus of the spinal segments T12-L2 (Fowler et al., 2008; Irving Nadelhaft & Vera, 1991; I. Nadelhaft et al., 1992). Sympathetic fibers pass along the splanchnic nerve, the hypogastric nerve and the inferior hypogastric plexus (Keast et al., 1990). Related to where sympathetic fibers terminate, they have different effects on the smooth muscles (Rohner Jr & Hannigan, 1980).the first type of sympathetic fibers that ends on the bladder body but not on the bladder neck releases norepinephrine as neurotransmitter that activates beta-adrenergic receptors and causes relaxation of the bladder smooth muscles. A different effect has the second type of sympathetic fibers that innervates mainly the bladder neck / internal urethral sphincter. They also release norepinephrine but here to activate alpha-adrenergic receptors that have a opposite effect and activate the smooth muscles. The regionally ordered expression of the different subtypes of adrenergic receptors allow the sympathetic pathway to maintain the sphincter mechanism by contraction and inhibit the detrusor at the same time, what is important for continence and normal lower urinary tract function.

Parasympathetic pathways: Parasympathetic preganglionic neurons are located in the lumbosacral spinal cord segments S2-S3 (in rats L6-S2), in the intermediolateral nucleus (lamina V and VII) (Petras & Cummings, 1978). The preganglionic fibers exit

through the ventral routs the spinal cord and travel along the pelvic plexus to terminate in the intramural ganglia (in rats in the major pelvic ganglion). They form synapses on postganglionic parasympathetic motoneurons that then innervate the entire bladder and bladder neck / internal urethral sphincter. Both pre- and post-ganglionic parasympathetic motoneurons are presumed to release cholinergic neurotransmitters and have excitatory effects on the smooth muscles (Park et al., 2006).

Somatic pathways: The external urethral sphincter muscle is directly innervated by somatic motoneurons located in Onuf`s nucleus (lamina IX) in the sacral spinal cord segments S1-S3 (Patel & Chapple, 2008) (in rats L5-L6 (McKenna & Nadelhaft, 1986)). The fibers exit the spinal cord by the ventral roots and travel by the pudendal nerve to the pelvic floor. They activate nicotine receptors on the striated muscles by the release of acetylcholine to cause contractions.

Afferent pathways: The hypogastric nerve, pelvic plexus and pudendal nerve are carrying afferent fibers from the lower urinary tract back to the spinal cord. Afferent fibers innervating the bladder are mainly following the pelvic plexus and hypogastric nerve, whereas the urethral sensation is conducted along the pudendal nerve. Like most other anatomical structures, the lower urinary tract innervated with two different afferent fiber types, A-delta and C-fibers. A-delta fibers are thinly myelinated and hence send their impulses with 2-30 m/s quite fast through the fibers with a 2 to 5 µm diameter (Basbaum et al., 2009). A-delta fibers conduct mainly information on sensation of pressure and respond to stretch of the bladder wall during filling. C-fibers are not myelinated and smaller in diameter (0.2-1.5 µm) and have hence a slower conduction velocity of less than 2 m/s. C-fiber endings respond to pain (very high intra-vesical pressure, i.e. >80mmHg), changes in temperature, chemical agents like capsaicin and menthol or inflammatory mediators (Basbaum et al., 2009). Interestingly, the response threshold of C-fibers can be modulated by e.g. inflammatory mediators in a fashion that they become activated at physiological intra-vesical pressures (Chuang et al., 2001). Lower urinary tract afferent fibers enter the spinal cord by the dorsal roots and follow the Lissauer`s tract in rostro-caudal directions. Nociceptive C-fibers terminate

beginning from the dorsal commissure and extend through Lamina I/II into Lamina V, the lateral edge of the dorsal horn (Birder & de Groat, 1993). After synaptic transmission of the nociceptive signal to interneurons, their fibers travel along the contralateral spinothalamic tract and end in the periaqueductal gray and thalamus (Nishii et al., 2008). Rats have interneurons in the sacral spinal cord that project directly to the pontine micturition center (B. F. M. Blok & Holstege, 2000) but it is not yet clear if humans and primates do have similar direct connections.

2.1.4 The central nervous system control of micturition

Spinal interneurons: Both excitatory and inhibitory interneurons involved in the lower urinary tract function have been identified by retrograde transneuronal labeling using pseurdorabies virus injections in to the bladder, urethra and external urethral sphincter. Pseudorabies virus labeled interneurons have been identified in the same regions that receive afferent input from the lower urinary tract: the dorsal commissure, Lamina I,II,V and VII just dorsal to where the preganglionic parasympathetic motoneurons lay (I. Nadelhaft & Vera, 2001; I. Nadelhaft et al., 1992). These findings have been confirmed by immediate early gene expression, c-fos after chemical stimulation of the bladder or urethra (Birder & de Groat, 1993; Birder et al., 1999). Interneurons form synapses to both sympathetic and parasympathetic preganglionic motoneurons or form long projections to the supraspinal centers, like the pontine micturition center, the periaqueductal gray and the thalamus and are involved in the supraspinal control of micturition. Some of the interneurons participate in spinal reflexes but its network is still not fully understood (de Groat et al., 1998; de Groat et al., 2015).

Projections from the brain to the spinal cord: Several brain regions involved in the lower urinary tract function have been identified in rats by retrograde transneuronal labeling using pseurdorabies virus injections in to the bladder, urethra and external urethral sphincter: pontine micturition center (Barrington`s nucleus), periaqueductal grey, serotonergic neurons of medullary raphe nuclei, noradrenergic neurons of the locus coeruleus, periventiricular medial preoptic and paraventricular nuclei of hypothalamus, dorsal thalamus, primary and secondary motor cortices and piriform cortices also

excibit virus-infected cells (Irving Nadelhaft & Vera, 1991; I. Nadelhaft & Vera, 2001; I. Nadelhaft et al., 1992; Vizzard et al., 1995). These findings have been confirmed using fMRI techniques with BOLD (blood oxygenation level dependent) signals in rats (Tai et al., 2009) and fMRI (DasGupta et al., 2007; Fowler & Griffiths, 2010) or PET-scan studies in humans (B. F. Blok et al., 1998).

A model of central nervous control of lower urinary tract: Both reflex circuitries controlling the lower urinary tract function contain neurons from the brain that activate and modulate the reflexes, interconnecting spinal cord interneurons, afferent signaling from the lower urinary tract to the spinal cord and efferent signaling from the spinal cord to the lower urinary tract. The lower urinary tract function consists of two main programs, the sympathetic mediated storage phase and the parasympathetic mediated voiding phase.

Sympathethic contribution to storage reflex: Sympathetic activity is heavily contributing to bladder storage function, i.e. the storage reflex and is mainly a spinal process. To prevent leakage during bladder filling (i.e. rise in intravesical pressure), urethral sphincter activity increases and the detrusor gets more and more relaxed. After pharmacological or surgical blocking of the sympathetic pathway, the urethral outflow resistance decreases, the bladder capacity is reduced and under constant volume conditions both frequency and amplitude of bladder contractions increase (de Groat et al., 2015). Sympathetic activity increases with rising bladder volume (and it is completely turned off during micturition). The sympathetic part of the storage reflex is generated by sacrolumbar spinal reflex pathways with inputs from pelvic plexus afferent fibers. Interneuron connections from afferent to sympathetic preganglionic motoneurons (e.g. intersegmental) are not yet fully understood (Beckel & Holstege, 2015).

External urethral sphincter contribution to storage and voiding reflex: Striated muscles of the external urethra sphincter are innervated by motoneurons from the sacral spinal pathway. The so called guarding reflex is active in two ways: On one hand a generally

increasing muscle tone in line with the increasing filling during storage phase (Thor & de Groat, 2010) and on the other hand rapid contraction of the external urethral sphincter in case of immediate extension of the bladder neck or pressure increase (i.e. coughing or lifting of heavy objects). The reflex is triggered by afferent input of the lower bladder and bladder neck. During micturition, the firing of external urethral sphincter motoneurons is inhibited and the guarding reflex is suppressed, most likely by inhibitory GABAergic and glycinergic interneurons located dorsal to the central channel that are directly driven by the pontine micturition center. This deactivation of the guarding reflex can be mimicked by electrical stimulation of the pontine micturition center (Kruse et al., 1991; Kruse et al., 1990) and is reduced in chronic spinal cord injured animals (i.e. detrusor sphincter dyssynergia). Hence, regulation of the guarding reflex is likely dependent on supraspinal control.

Parasympathetically mediated voiding reflex including the pontine micturition center and the periaqueductal gray: In contrast to the storage reflex that is mainly a spinal mediated process, the voiding reflex is controlled by suprasacral structures, essentially by the pontine micturition center, a cell group located in the dorsolateral tegmentum of the pons. Electrical or chemical stimulation of the pontine micturition center in cats lead to immediate relaxation of the urethra and with a delay of two seconds to contraction of the detrusor (Holstege et al., 1986). These findings have been confirmed in human PET-scan studies showing increased blood flow in the pontine micturition center associated with micturition in male (B. F. Blok et al., 1997) and female healthy volunteers (B. F. Blok et al., 1998). The pontine micturition center has direct monosynaptic connections to the preganglionic parasympathetic motoneurons that can elicit bladder contraction. Main excitatory neurotransmitter of this pathway is glutamate acting on NMDA and non-NMDA receptors (Matsumoto et al., 1995). Additional inhibitory receptors for serotonin and corticotropin releasing factor have been identified on preganglionic parasympathetic motoneurons (Wood et al., 2009). The corticotropin releasing factor has been identified as selective target to immunohistochemically stain the cells of the pontine micturition center and its fibers

(Valentino et al., 2011). In summary, the pontine micturition center gets direct afferent input from the lower urinary tract by sacral lamina I/II interneurons, it has direct monosynaptic excitatory glutamatergic connections to preganglionic parasympathetic motoneurons that elicit detrusor contraction as well as direct monosynaptic connections to inhibitory GABAergic and glycinergic interneurons positioned dorsal to the central channel that inhibit the guarding reflex, i.e. external urethral sphincter motoneurons. The pontine micturition center orchestrates voiding (Michels et al., 2015). The periaqueductal grey is constantly informed by afferent signals from the spinal cord on the filling stage of the bladder (Tai et al., 2009). It is the most important input with direct excitatory fibers to the pontine micturition center and is able to elicit micturition (Beckel & Holstege, 2015). The periaqueductal gray together with the pontine micturition center are therefore believed to act as brainstem micturition switch.

Figure 2

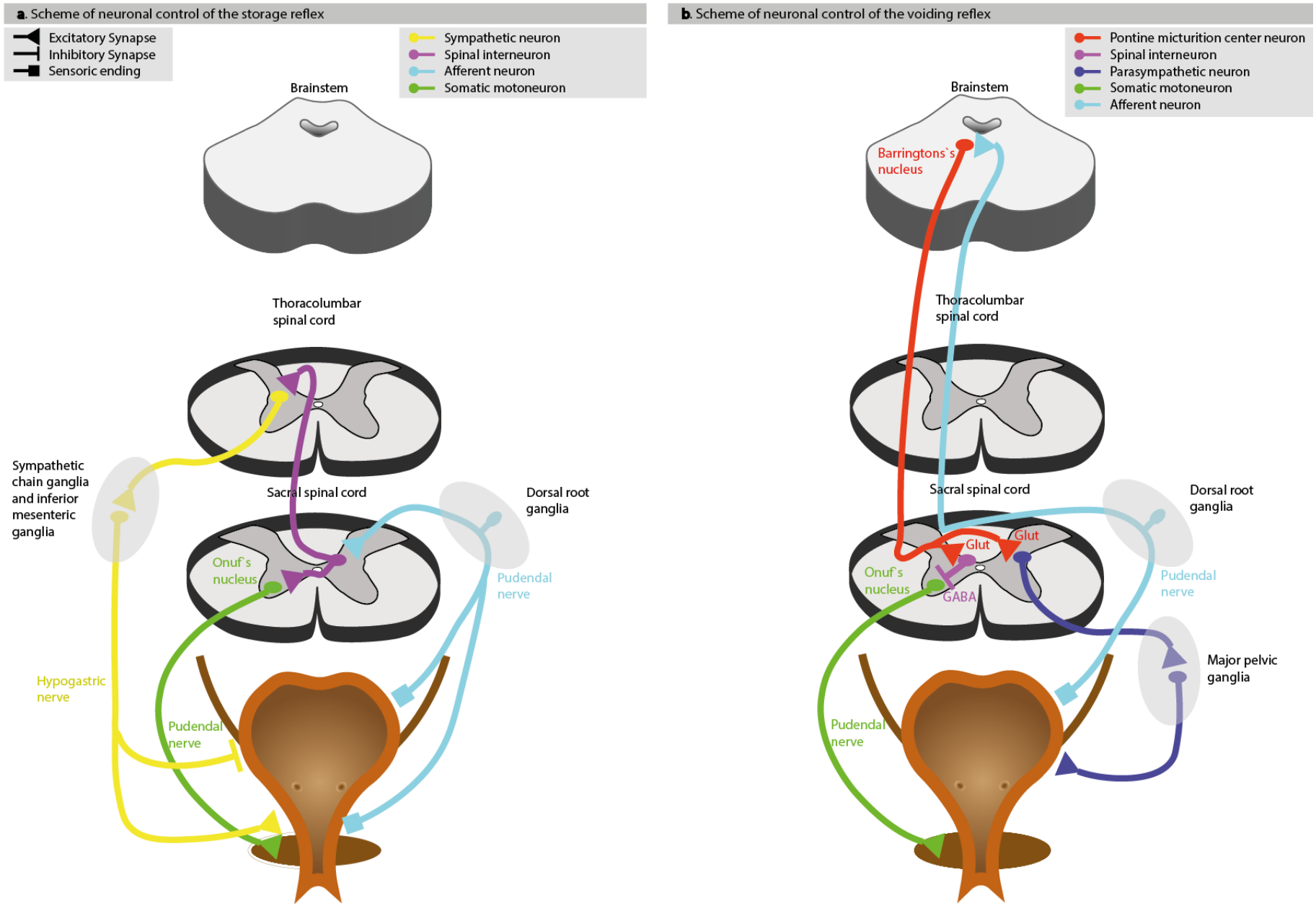

a. Scheme of neuronal control of the storage reflex
Excitatory Synapse
Inhibitory Synapse
Sensoric ending
Sympathetic neuron
Spinal interneuron
Afferent neuron
Somatic motoneuron
Brainstem
Thoracolumbar spinal cord
Sympathetic chain ganglia and inferior mesenteric ganglia
Sacral spinal cord
Dorsal root ganglia
Onuf`s nucleus
Pudendal nerve
Hypogastric nerve
Pudendal nerve
b. Scheme of neuronal control of the voiding reflex
Pontine micturition center neuron
Spinal interneuron
Parasympathetic neuron
Somatic motoneuron
Afferent neuron
Brainstem
Barringtons`s nucleus
Thoracolumbar spinal cord
Sacral spinal cord
Dorsal root ganglia
Glut
Glut
Onuf`s nucleus
GABA
Pudendal nerve
Major pelvic ganglia
Pudendal nerve

Figure 3: Scheme of the neuronal control of storage and voiding reflex; **a.** Detailed scheme of the spinal neuronal circuitry involved in the sympathetic mediated storage reflex. **b.** Detailed scheme of the pontino-spinal neuronal circuitry involved in the parasympathetic mediated voiding reflex.

Superordinate control of micturition: The periaqueductal gray receives input from superordinate brain centers, allowing for deliberate initiation or retarding of micturition. Meta-analyses of PET-studies have identified the following brain regions being activated during storage phase: the periaqueductal gray, the pons, the medulla, the thalamus, the insula, the anterior cingulate, the prefrontal cortex and the supplementary motor area (DasGupta et al., 2007). Whereas micturition during micturition the following regions were activated: the periaqueductal gray, the hypothalamus, the insula and the prefrontal cortex (B. F. Blok et al., 1998; B. F. Blok et al., 1997; DasGupta et al., 2007). The different brain regions and their specific functions are still not fully understood and there are currently many functional brain imaging studies ongoing to unscramble this network, particularly to investigate brain regions involved in different types of lower urinary tract dysfunction (Leitner et al., 2014; Walter et al., 2014).

2.1.5 References

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2.2 Neurogenic lower urinary tract dysfunction in spinal cord injured patients

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Further contributions by:

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M.P.S.: Produced all the figures. Wrote the manuscript (edited by T.M.K.).

2.2.1 Abstract

Spinal cord injury (SCI) is a devastating event with far-reaching consequences for the individual's health and the family's economic and social future. Most of these patients will develop neurogenic lower urinary tract dysfunction (NLUTD), which has a highly negative impact on patients' health-related quality of life and may progressively lead to end stage renal failure. In addition, complications such as urinary tract septicemia are life-threatening. Modern proactive neuro-urological management aiming at the preservation of upper urinary tract function, the control of urinary tract infection, and the maintenance of a low-pressure bladder that is both continent and capable of complete emptying has revolutionized the treatment of SCI patients. Indeed, in the past, renal disease was responsible for almost 50% of deaths in SCI patients. This changed dramatically and nowadays urinary disease accounts for only about 13% of deaths in SCI patients. Nevertheless, lower urinary tract function remains one of the most important issues in health and general life of a SCI patient.

2.2.2 Incidence and epidemiology of spina cord injury in Switzerland

The incidence of traumatic spinal cord injury in Switzerland is 18.0 (95% confidence interval 16.9-19.2) per one million population per year (Chamberlain et al., 2015). This is around the average value among other European countries ranging from 8.3 in Denmark (Bjornshave Noe et al., 2015) to 33.6 per million per year in Greece (Divanoglou & Levi, 2009). The incidence in Switzerland is rather low compared to USA, where the incidence rate is 53 per one million population per year (Jain et al., 2015). This may be explained by much higher numbers of motor vehicle crashes and firearm injuries. From a Swiss cohort of 932 cases collected from 2005 to 2012 (Chamberlain et al., 2015), it is reported that about 75% are male patients and fall is with 37% the most common cause (causes in decreasing likelihood: tree/off-ladder; window/balcony/roof; tripped -stairs), followed by sports with 27% (causes in decreasing likelihood: skiing/snowboarding; paragliding; swimming/diving; motocross; horseback riding) and transport with 23% (causes in decreasing likelihood: car crash; motorcycle crash; bicycle crash). The most common level of injury is between T1 and S5 (about 30%) and the most common lesion is American Spinal Injury Association (ASIA) impairment scale (AIS) (Kirshblum et al., 2011) D, i.e. AIS D (about 40%). Swiss SCI patients are in average young with a mean age of 48 years (tetraplegia 53.5, paraplegia 43.8) at onset of disease and with the biggest patient group in age between 16 to 30 years. Estimated life expectancies calculated from an Australian SCI population, range depending on age of SCI patient at injury of 25 to 65 from 38.7 to 12.1 years and are markedly reduced compared to age matched healthy population with estimated life expectancies of 55.7 to 19.1 years (Middleton et al., 2012). Nowadays, however, patients with SCI have a rather high life expectancy leading to a high total number of SCI patients despite a rather low incidence rate.

2.2.3 Consequences of a spinal cord injury

In 1970`s about 43% of SCI patients died because of renal failure, it was the major cause of death at that time (Hackler, 1977). And, this number did not incorporate SCI patients dying because of septicemia, another very common complication of neurogenic lower urinary tract dysfunction (NLUTD). Hence the true number of SCI patients dying of any complication of NLUTD is likely to have been even higher at that time. Thanks to modern neuro-urological assessment and care, the mortality rate has drastically reduced to about 13% up to the year 2000 (Lidal et al., 2007) and will nowadays be even lower (Schops et al., 2015). However, there are currently only symptomatic treatments available, e.g. the current gold standard treatment is four to six times daily self-catheterization (Blok et al., 2016; Groen et al., 2016; Panicker et al., 2015) what may lead to common complications such as urinary tract infections, strictures and urethral perforations and also emerges into massive social and economic costs for both the patient and the society. Intermittent self-catheterization, however, is not appropriate for each patient (for instance a tetraplegic patient with impaired manual dexterity may need an indwelling catheter or a health care provider performing third party intermittent catheterization). Importantly, intact bladder, sexual and bowel function remain among the highest rehabilitation priorities of SCI patients (Simpson et al., 2012). In this context it is of great value to predict bladder outcomes so that we have recently derived and validated two models predicting urinary continence and complete bladder emptying 1 year after injury (Pavese et al. 2016): The full model is based on lower extremity motor score (LEMS), light-touch sensation in the S3 dermatome, and the Spinal Cord Independence Measure (SCIM) subscale respiration and sphincter management within the first 40 days of SCI; the simplified model is based on LEMS only. Early prediction of bladder outcomes may optimize counseling and patient-tailored rehabilitative interventions and improve patient stratification in future clinical studies.

2.2.4 Assessment of neurogenic lower urinary tract dysfunction and definition of its different types

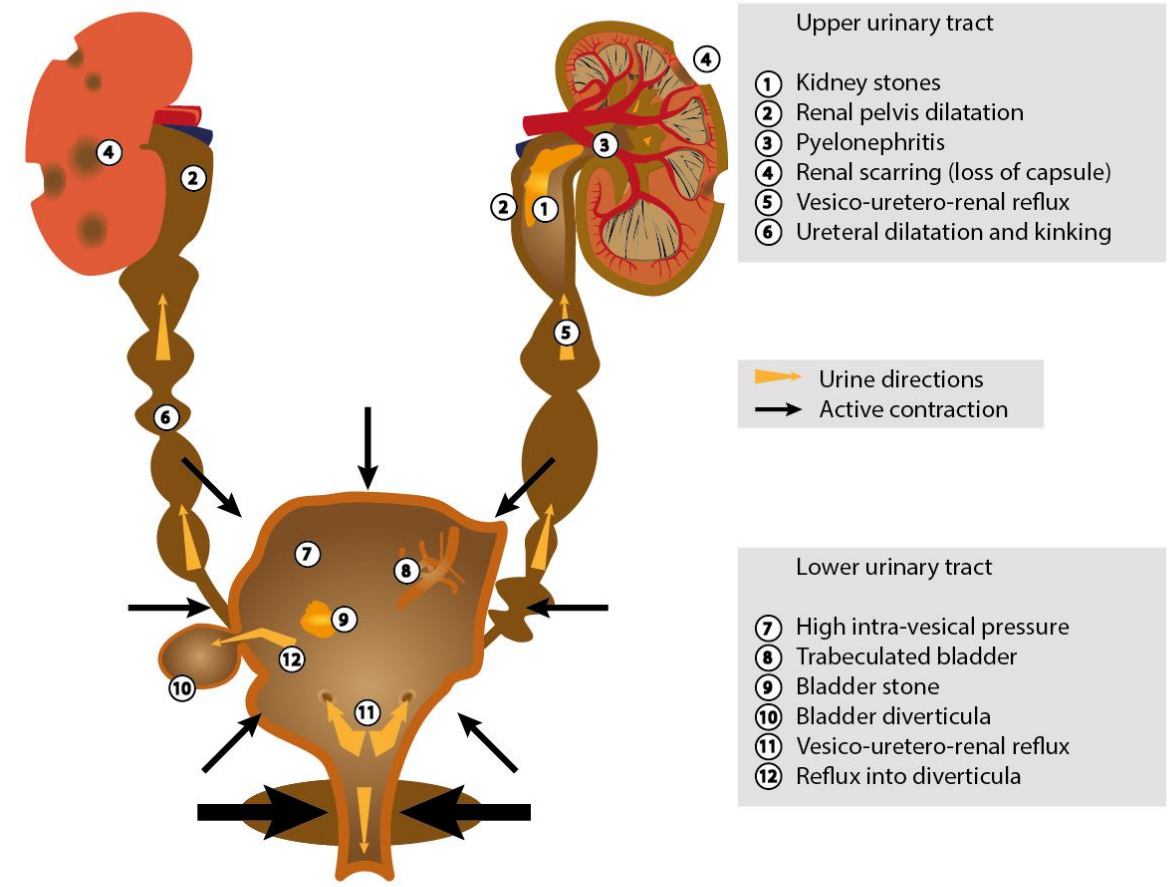
NLUTD is defined as any type of lower urinary tract dysfunction with and underlying neurological cause. The gold standard to assess NLUTD are video-urodynamics (For details please see chapter 2.3: Urodynamic management of the spinal cord injured patient).

Here the most common subtypes of NLUTD as defined by the International Continence Society (ICS) (Abrams, 2003):

> *Overactive bladder syndrome* is defined as “urgency, with or without urgency incontinence, and usually with frequency and nocturia”.
>
> *Detrusor overactivity* is defined as “a urodynamic observation characterized by involuntary detrusor contractions during the filling phase that may be spontaneous or provoked. Detrusor overactivity is subdivided into idiopathic detrusor overactivity and neurogenic detrusor overactivity”.
>
> *Detrusor underactivity* is defined as “a contraction of reduced strength and/or duration, resulting in prolonged bladder emptying and/or failure to achieve complete bladder emptying within a normal time span”.
>
> *Detrusor sphincter dyssynergia* is defined as “a detrusor muscle contraction concurrent with an involuntary contraction of the external urethral sphincter muscle, and occasionally flow may be prevented altogether”.

Figure 3

a. Scheme of detrusor sphincter dyssynergia and long term consequences



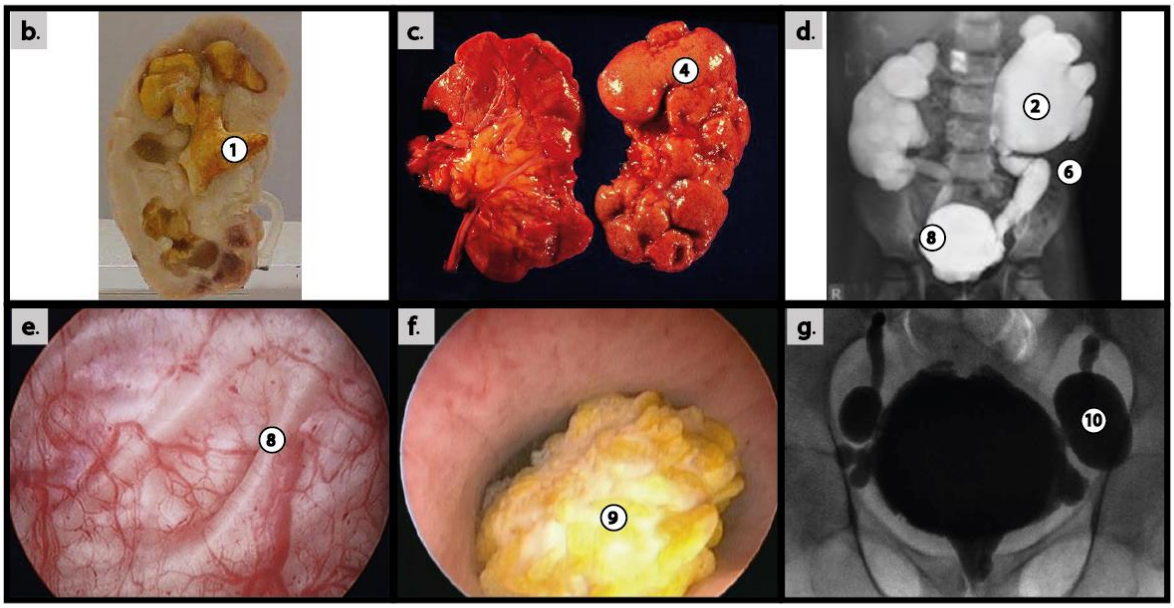


Figure 3: a. Scheme of detrusor sphincter dyssynergia and its long-term consequences. **b.** Large kidney stone (from: https://www.healthtap.com/user_questions/215154) **c.** severe renal scarring (from: http://body-disease.com/pyelonephritis-acute/) **d.** severe grade four vesico-uretero-renal reflux from video-urodynamic assessment. **e.** Bladder trabeculation (from https://www.centerforreconstructiveurology.org/urethral-stricture/causes-symptoms/) **f.** bladder stone (from http://ourologos-chania.gr) **g.** “Mikey mouse” bladder with bladder diverticula, bilateral vesico-uretero-renal reflux and detrusor sphincter dyssynergia.

2.2.5 Neurogenic lower urinary tract dysfunction after spinal cord injury

Most SCI patients suffer from NLUTD (Schops et al., 2015). Importantly, ambulatory and nonambulatory patients with acute SCI have similar risk of unfavorable urodynamic measures (Bellucci et al., 2013), i.e. urodynamic investigations are strongly recommended in all acute SCI patients independent of the ability to walk. The most common and dangerous type of NLUTD is detrusor sphincter dyssynergia that occurs in more than 90 percent of SCI patients (Weld et al., 2000). Detrusor sphincter dyssynergia lead to an increase of intra-vesical pressure which may result in vesico-uretero-renal reflux causing pendulum volumes. Ureter and pelvicaliceal system may become dilated storing large urine volumes, and during voiding this oscillating volume will contribute to high post void residual which is a risk factor for urinary tract infection including ascending pyelonephritis and life-threatening urinary tract septicemia. High intra-vesical pressure can also lead to morphological changes of the bladder wall such as trabeculations and (pseudo-) diverticula favoring urinary tract infection. Bladder wall thickening as a result of detrusor hypertrophy may cause obstruction at the vesico-ureteral junction and result in reduced bladder compliance. Finally, without appropriate treatment, NLUTD often leads to vicious circle with highly relevant impairment of quality of life and with risk for the upper urinary tract that may even result in end-stage renal disease.

Type and severity of NLUTD depends on the site and nature of the SCI (Panicker et al., 2015; Weld & Dmochowski, 2000) (See also Figure 6). Acute SCI leads to a state named “spinal shock”, characterized by detrusor acontractility/hypocontractility and voiding dysfunction upon complete urinary retention, which in case of a suprasacral lesion (today the vast majority of spinal cord injuries) is followed by slow development of detrusor overactivity mostly combined with detrusor sphincter dyssynergia. These dysfunctional patterns are probably caused by C-fiber-mediated spinal reflex pathways, possibly related to the interrupted regulatory mechanism between the lower

urinary tract and midbrain for urine storage and voiding. Voiding dysfunction due to detrusor hypo-/acontractility or detrusor sphincter dyssynergia is generally managed by intermittent self-catheterization. First-line treatment for subsequent detrusor overactivity are antimuscarinics. In the case of refractory detrusor overactivity, intradetrusor onabotulinumtoxinA injections have become an established and highly effective therapeutic option (Leitner et al., 2016).

Figure 4

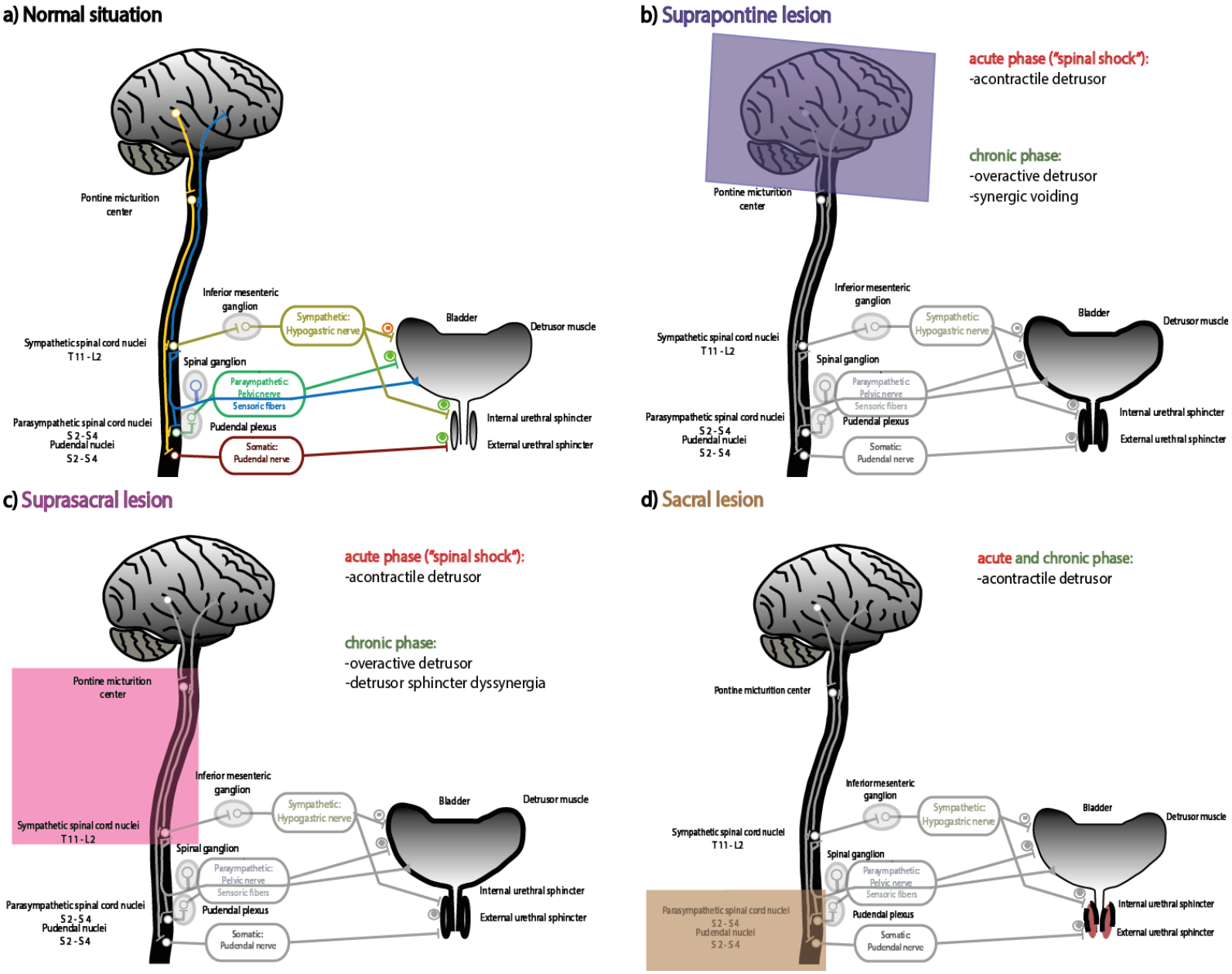

a) Normal situation
Pontine micturition center
Inferior mesenteric ganglion
Sympathetic: Hypogastric nerve
Bladder
Detrusor muscle
Sympathetic spinal cord nuclei T11 - L2
Spinal ganglion
Paraympathetic: Pelvic nerve
Sensoric fibers
Parasympathetic spinal cord nuclei S2 - S4
Pudendal nuclei S2 - S4
Pudendal plexus
Somatic: Pudendal nerve
Internal urethral sphincter
External urethral sphincter
b) Suprapontine lesion
acute phase ("spinal shock"):
-acontractile detrusor
chronic phase:
-overactive detrusor
-synergic voiding
Pontine micturition center
Inferior mesenteric ganglion
Sympathetic: Hypogastric nerve
Bladder
Detrusor muscle
Sympathetic spinal cord nuclei T11 - L2
Spinal ganglion
Paraympathetic Pelvic nerve
Sensoric fibers
Parasympathetic spinal cord nuclei S2 - S4
Pudendal nuclei S2 - S4
Pudendal plexus
Somatic: Pudendal nerve
Internal urethral sphincter
External urethral sphincter
c) Suprasacral lesion
acute phase ("spinal shock"):
-acontractile detrusor
chronic phase:
-overactive detrusor
-detrusor sphincter dyssynergia
Pontine micturition center
Inferior mesenteric ganglion
Sympathetic: Hypogastric nerve
Bladder
Detrusor muscle
Sympathetic spinal cord nuclei T11 - L2
Spinal ganglion
Paraympathetic: Pelvic nerve
Sensoric fibers
Parasympathetic spinal cord nuclei S2 - S4
Pudendal nuclei S2 - S4
Pudendal plexus
Somatic: Pudendal nerve
Internal urethral sphincter
External urethral sphincter
d) Sacral lesion
acute and chronic phase:
-acontractile detrusor
Pontine micturition center
Inferior mesenteric ganglion
Sympathetic: Hypogastric nerve
Bladder
Detrusor muscle
Sympathetic spinal cord nuclei T11 - L2
Spinal ganglion
Paraympathetic Pelvic nerve
Sensoric fibers
Parasympathetic spinal cord nuclei S2 - S4
Pudendal nuclei S2 - S4
Pudendal plexus
Somatic Pudendal nerve
Internal urethral sphincter
External urethral sphincter

Figure 4: Lesion level and corresponding lower urinary tract dysfunctional pattern. **a.)** Normal situation. **b.)** Suprapontine lesion with acontractile detrusor in the acute phase and overactive detrusor with synergic voiding in the chronic phase. **c.)** Suprasacral lesion with acontractile detrusor in the acute phase and overactive detrusor with detrusor sphincter dyssynergia in the chronic phase. **d.)** Sacral lesion with acontractile detrusor in the acute and chronic phase.

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2.3 Urodynamic management of the spinal cord injured patient

by **Marc P. Schneider**

Further contributions by:

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M.P.S.: Produced all the figures. Wrote the manuscript (edited by T.M.K.).

2.3.1 Introduction

Urodynamic investigation (UDI) is the only method that can objectively assess the function and dysfunction of the lower urinary tract (LUT) (Groen et al., 2016). Considering that most patients with spinal cord injury (SCI) suffer from LUT dysfunction, it is generally agreed that UDI is the gold standard to evaluate urinary tract function. Indeed, UDI allows for a patient-tailored management and regular follow-up with UDI seems beneficial (Schops et al., 2015). Remarkably, ambulatory and non-ambulatory patients with acute SCI have similar risk of unfavorable urodynamic measures so that complete neuro-urological assessment including UDI is strongly recommended in all acute SCI patients independent of the ability to walk (Bellucci et al., 2013). Same session repeat UDIs are crucial in clinical decision making, since repeat measurements may yield completely different results (Bellucci et al., 2012). Any technical source of artifacts must be critically considered and all urodynamic findings have to be reported in detail: Maximum storage detrusor pressure, bladder compliance, first sensation of bladder filling, first and strong desire to void, urgency, urinary leakage or maximum cystometric capacity reflect the storage phase and maximum flow rate, voided volume, voiding time or average flow rate mirror the voiding phase and pelvic floor electromyography is assessing both the storage and voiding phase,. Urodynamic values in combination with the bladder diary and the medical history allow for a diagnosis and future treatments. Special attention should be given to high standardization of the urodynamic technique, since this is the prerequisite for reproducible and reliable result. Hence, UDI has to be performed and reported in accordance with the standards of the International Continence Society (ICS) (Abrams et al., 2002; Gammie et al., 2014).

2.3.2 Cystometry (cystomanometry) and pressure-flow study

Cystometry (cystomanometry): It is performed to assess the storage (filling) phase using a double-lumen transurethral or suprapubic (6-10 French) catheter (the catheter lubricant should be without anesthetic additive to avoid an impact on bladder sensation). The bladder has to be emptied before each UDI. A physiological filling rate (it should ideally not exceed body weight in kg divided 4 (Gammie et al., 2014)) should be used with physiological saline or a mixture with contrast medium at body temperature since fast filling, different ion concentrations and low temperature may be provocative affecting urodynamic results (Blok et al., 2016). During filling, provocation tests including coughing, change of position from supine or sitting to standing or handwashing, can be used to demonstrate inducible detrusor overactivity, urgency or stress urinary incontinence. It is highly recommended to repeat UDI at least once, since repeat measurements may yield completely different results (Bellucci et al., 2012). Ice water testing can be used to test for a temperature-sensitive reflex detrusor contraction mediated by afferent C-fibers. Detrusor overactivity may be demonstrated even if there is no detrusor activity in the standard UDI helping to unmask a putatively acontractile detrusor. Since the ice water test is an unphysiological investigation that may relevantly bias subsequent UDI it should be performed at the end and not precede more physiological standard UDI (Kozomara et al., 2015). Bladder sensation during UDI is assessed on the basis of the volume in the bladder at patients' first sensation of bladder filling, first desire to void and strong desire to void. Urgency is defined as sudden compelling desire to void (Abrams et al., 2002).

Pressure-flow study: It is performed to assess the voiding (emptying) phase and reflects the co-ordination between detrusor and urethra / pelvic floor during micturition. Possible pathological findings include detrusor hypo-/acontractility, bladder outlet obstruction including DSD and post void residual. It has to be considered that many patients with SCI will not be able to void spontaneously, i.e. maximum cystometric capacity and post void residual will be identical.

2.3.3 Surface electromyography

Electromyography (EMG) of the pelvic floor including urethral and anal spincter activity, is an established method for the diagnosis of bladder sphincter dysfunctions. Combined pelvic floor EMG and videourodynamics are the most acceptable and widely agreed methods for diagnosing DSD, and they are superior to external urethral sphincter pressure measurement (Suzuki Bellucci et al., 2012). The pelvic floor EMG is usually simultaneously measured with cystometry. Surface electrodes should be placed ventral and close to the anus. The EMG amplitude is measured in mV (millivolts) and provides a simple semi-quantitative tracing of the muscle activity over time. It has to be considered that after changing of the electrodes position (for instance UDI at a follow-up visit), the amplitudes of the different EMG measurements cannot be compared because of by different electrode impedances and different muscle masses between the electrodes. However, the shape of the tracing will remain the same and thus comparable. Surface electrodes are therefore highly vulnerable to artefacts and the signal should be monitored throughout the whole measurements (e.g. by zoom tracing on the tracking software, oscilloscope or audio signal), in particular lying position in combination with urine leakage leading to bypass the electrodes and mimicking DSD (defined by the ICS as a detrusor contraction concurrent with an involuntary contraction of the urethral and/or periurethral striated muscle (Abrams et al., 2002)) can become a problem.

2.3.4 Videourodynamics

Videourodynamics is the combination of cystometry and pressure-flow study (in the case that spontaneous voiding is possible) with imaging. It is the gold standard for UDI in neuro-urological disorders, especially in patients with SCI. If this is not available, then cystometry continuing into a pressure-flow study should be performed with cystography. Videourodynamics allow to detect vesico-uretero-renal reflux, bladder trabeculation, pseudo-diverticula, diverticula, detrusor internal sphincter dyssynergia (DSID, bladder neck dyssynergia), detrusor external sphincter dyssynergia and influx

into the seminal vesicles and prostate. However, ionizing radiation should be kept to a minimum according to the as low as reasonable achievable (ALARA) principle.

2.3.5 Safety

The main risks of UDI are associated with urethral catheterization. If patient`s sensation is preserved, dysuria is quite common on the first days following UDI. In patients with impaired bladder and urethral sensation, the risk for urethral lesion during catheterization is increased due to lack of alarming pain, e.g. by via falsa.

Prophylactic antibiotics reduced the risk of bacteriuria but not of UTIs after UDIs (Foon et al., 2012). Thus, anbitiotic prophylaxis is not generally recommended, especially taking into account the alarming antibiotic resistance worldwide

A relevant issue in SCI patients, particular in those with a lesion at or above T6, is UDI induced autonomic dysreflexia (Blackmer, 2003) with an overall incidence of up to 73% (Walter et al., 2016). Thus, (if available continuous) cardio-vascular monitoring during UDI is strongly recommended. In the case of autonomic dysreflexia during examination, stopping UDI and immediate emptying of the bladder is mandatory to avoid life-threating situation in daily clinical practice (in special situations antihypertensive treatment might become necessary, for instance nifedipine) (Groen et al., 2016; Walter et al., 2016).

Detailed history on potential allergies is imperative especially considering the allergic potential of latex gloves and catheters or contrast medium.

2.3.6 Description of urodynamic findings

UDI assesses both the storage (filling) and voiding (emptying) phase resulting in several observations and values (Table 1). In addition, filling rate and temperature, different sensation qualities (details see Table 1), urological medications, former treatments and position of the patient during examination have to be reported.

Considering the urodynamic tracings and all above-mentioned observations / values, a urodynamic diagnosis can be established consisting of bladder sensation (details see Table 1), bladder capacity (normo ~350-550 mL, hypo ~<350 mL or hyper ~>550 mL), detrusor function (details see Table 1) and sphincter function (details see Table 1).

Table 1 – Urodynamic observations and values

Urodynamic observations (adapted from Abrams et al 2002)
Intravesical pressure [cmH2O]
Abdominal pressure [cmH2O]
Detrusor pressure [cmH2O]
Filling rate (physiological is defined as less than - predicted body weight in kg divided by 4) [mL/min]
Bladder sensation (First sensation of bladder filling, first desire to void, strong desire to void; Normal-, increased-, reduced-, absent-, non-specific bladder senstaion, bladder pain or urgency)

Storage
Bladder compliance (volume change divided by the change in detrusor pressure) [mL/cmH2O]
Maximum cystometric capacity [mL]
Abdominal leak point pressure [cmH2O]
Detrusor leak point pressure [cmH2O]
Maximum detrusor pressure durring storage phase [cmH2O]

Voiding
Flow rate [mL/s]
Flow time [s]
Voided volume [mL]
Voiding time [s]
Average flow rate [mL/s]
Maximum detrusor pressure during voiding phase [cmH2O]
Maximum detrusor pressure at maximum flow [cmH2O]
Post void residual [mL]

Urodynamic diagnosis:
Detrusor function
Normal detrusor function
Detrusor overactivity
Phasic detrusor overactivity
Terminal detrusor overactivity
Detrusor overactivity incontinence
Acontractile / hypocontractile (underactive) detrusor
Normal bladder compliance
Low bladder compliance
Normal bladder capacity
Low bladder capacity
Stress urinary incontinence

Sphincter function
Bladder outlet obstruction
Detrusor sphincter dyssynergia
Underactive / overactive sphincter

2.3.7 Indication for urodynamics in SCI patients

It is important to note that early UDI may be useful even in patients with incomplete SCI who have preserved/recovered significant function as early treatment (if indicated) may improve long-term outcomes. Indeed, patients who are ambulatory after SCI also have high risk urodynamic changes on UDI (Bellucci et al., 2013; Patki et al., 2006). Thus, first UDI should be performed within 3 months after SCI. In patients with unfavorable urodynamic parameters (i.e. high maximum detrusor pressure during the storage phase (>40cmH2O) (McGuire et al., 1981), low compliance bladder (<20mL/cmH2O) (Hackler et al., 1989)), detrusor overactivity, DSD and vesico-uretero-renal reflux), follwow-up UDI should be considered 1-3 months after initation of treatment (such as antimuscarinics, intradetrusor onabaotulinumtoxinA injections etc.) to assess the treatment effect.

2.3.8 Trouble shooting and good urodynamic practice

Abdominal, rectal and/or detrusor contractions can increase intravesical pressure (Pves) in a similar way. Thus, to improve quality and reliability of urodynamic assessments, it is essential to monitor both Pves and the abdominal pressure (Pabd) simultaneously resulting in the detrusor pressure (Pdet=Pves-Pabd), so that all 3 tracings should be recorded for trouble shooting. Pressure changes of the detrusor are smooth. whereas changes in Pves and Pabd are usually sharp as the consequence of a sudden pressure increase to coughing which is used to check for proper pressure transmission and should be frequently repeated (Schafer et al., 2002) However, please consider that depending on the lesion level and completeness of injury, SCI patients may not be able to cough. Initial Pabd and Pves should be in an expected range and initial Pdet should be close to zero. Resting values are vulnerable to position changes of the patient. Fast filling can mimic a low compliance bladder, hence use physiological speed and stick to the formula for calculation of filling speed (Gammie et al., 2014).

Other common problems are a negative Pdet (Gammie et al., 2014), potentially due to a too high Pabd (consider gently repositioning of the rectal balloon or draining some of rectal balloon filling) or a too low Pves (first check for potential air bubble trapped in the catheter or catheter may be kinked and blocked, can be tested by slowly flushing of the catheter or second, the catheter needs to be repositioned), a too high initial Pdet (normal empty Pdet is between 0-5 cmH_2O and any value higher than 10 cmH_2O needs to be checked (Gammie et al., 2014): check if Pves and Pabd are in the expected range, the zero balance and proper signal response to coughing. If Pabd is too low, very slowly flush the rectal balloon with 1-2 mL, if Pabd is too high (same procedure as explained above) or a too high Pves (check catheter placement and for kinking, solution may be catheter repositioning and slowly flushing the catheter).

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2.4 Anti-Nogo-A antibody: A treatment option for neurogenic lower urinary tract dysfunction?

by **Marc P. Schneider**

Further contributions by:

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M.P.S.: Wrote the manuscript (edited by T.M.K. and M.E.S.).

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In the late 1980's, Caroni and Schwab (Caroni P. & Schwab, 1988) showed that the myelin membrane of oligodendrocytes is inhibiting nerve fiber growth in the central nervous system (CNS). A monoclonal IgM antibody against an unknown CNS myelin protein later known as Nogo-A induced substantial axonal sprouting and functional recovery in vitro and in vivo. Nowadays, the responsible neurite growth inhibitory surface protein Nogo-A and its receptors NgR1 and S1PR2 are identified and well-studied (Kempf et al., 2014; Schwab & Strittmatter, 2014). Nogo-A destabilizes the cytoskeleton via the rho/ROCK pathway causing growth cone collapse and inhibits neuronal growth and plasticity by down-regulation of growth-associated genes. Nogo-A suppression or neutralization leads to an increase in sprouting, axonal regeneration and neuronal plasticity and thereby to greater functional recovery after different types of CNS injuries.

In close collaboration with Novartis, a function blocking, high affinity human anti-human Nogo-A antibody (ATI355) was developed for intrathecal application. A clinical phase 1 study using this anti-Nogo-A antibody in acute, severe spinal cord injury patients was conducted by Novartis in several spinal cord injury centers in Europe and Canada. This phase 1 safety study has been recently completed successfully (http://clinicaltrials.gov/show/NCT00406016) and a placebo-controlled phase 2 "proof of concept study" is in preparation. In addition, based on very promising findings in animal studies (Schwab & Strittmatter, 2014; Wahl et al., 2014), trials assessing the effect of anti-Nogo-A in acute stroke and in amyotrophic lateral sclerosis (conducted by GSK) are in preparation or on-going (Meininger et al., 2014). Importantly, anti-Nogo-A antibody treatment might also become an effective therapeutic option for neurogenic lower urinary tract dysfunction (NLUTD): Liebscher et al. (Liebscher et al., 2005) have found a significantly higher rate of corticospinal tract sprouting and regeneration after transection in adult rats when the animals were treated with function blocking antibodies against the neurite growth inhibitory protein Nogo-A as compared to control antibody treated rats (Figure). The treated animals reached significantly higher scores in a variety of sensory-motor tests and showed improved recovery of locomotion and

motor coordination. During the first ten days after injury, the animals were not able to void and their bladders had to be emptied manually two to three times a day. In the control antibody treated group, voiding started to recover on average around 24 days after SCI. Remarkably, voiding was restored more than one week earlier in the anti-Nogo-A antibody treated rats (Liebscher et al., 2005).

Suppression of Nogo-A or its receptor NgR1 enhances neurite growth in the adult CNS (Schwab, 2010; Schwab & Strittmatter, 2014). In the injured CNS, regenerative and compensatory sprouting as well as long distance regeneration of fibers in many parts of the spinal cord and brain are enhanced by functional blockade of Nogo-A signaling(Schwab & Strittmatter, 2014). These processes probably lead to new connections and functional circuits, for example from the pontine micturition center to the sacral micturition neurons, directly or via long proprio-spinal interneurons. In addition, anti-Nogo-A antibodies could induce plasticity in the circuits of the pontine and sacral micturition centers causing reorganization.

To elucidate the mechanisms of action and the potential of anti-Nogo-A antibody therapy for treating NLUTD, animal studies with detailed urodynamic measurements in different neuronal disease models causing NLUTD are currently ongoing. In addition, urodynamic investigations are planned to assess lower urinary tract function in the coming clinical studies. Future animal and human studies will show if anti-Nogo-A antibody treatment has the potential to improve our management of NLUTD.

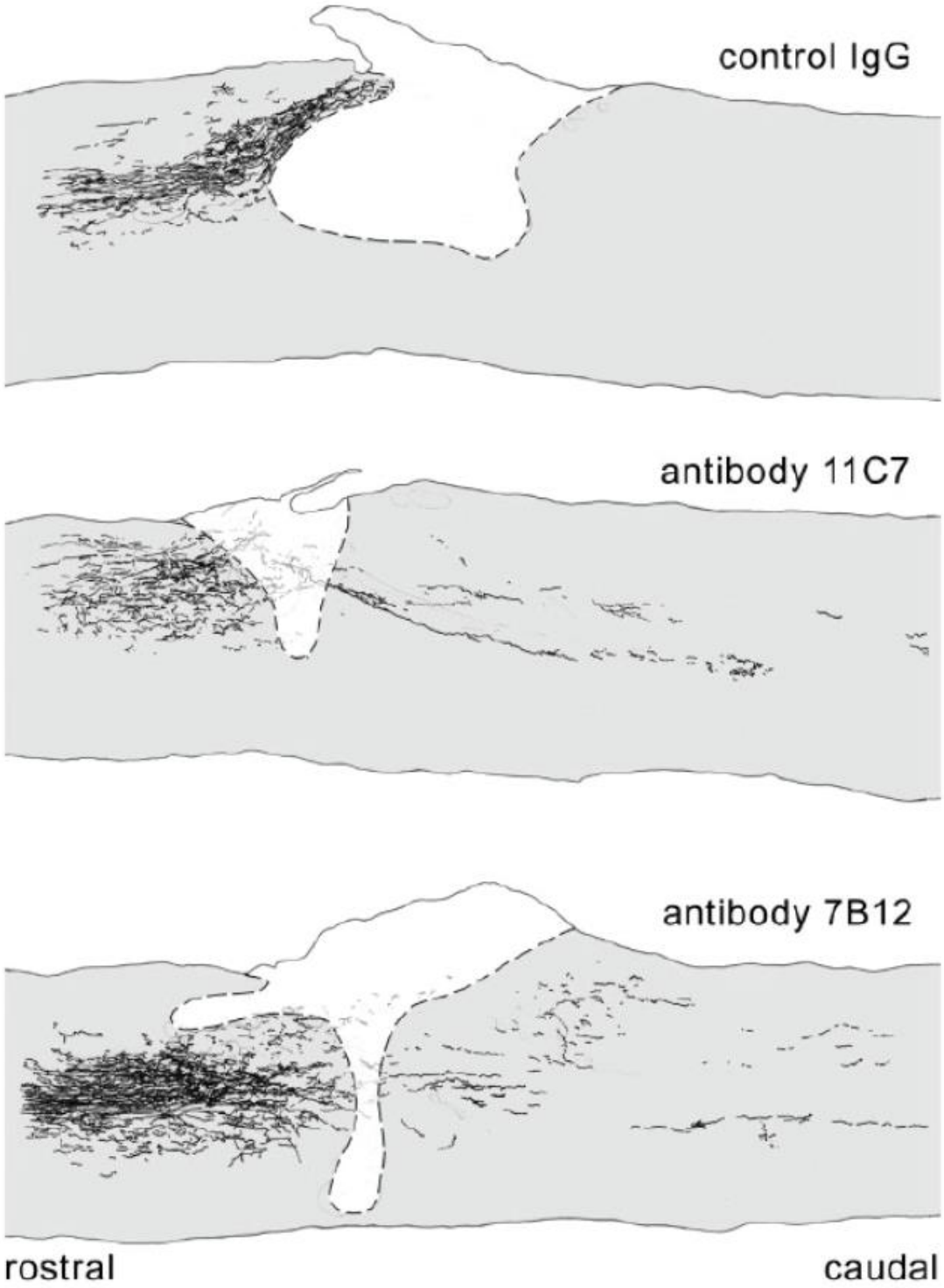
control IgG
antibody 11C7
antibody 7B12
rostral
caudal

Figure 5: Spinal cord injured rats were treated with two different IgG anti-Nogo-A antibodies (11C7 and 7B12). Reconstructions of the spinal hemicord with labeled corticospinal tract (CST), lesion site (light area), rostral (left side) sprouting zone, and CST fibers regenerating over tissue bridges (gray-shaded depiction) into the caudal spinal cord (right side). In both anti-Nogo-A antibody treated groups (11C7 and 7B12) were substantially more CST fibers regenerating (dark fibers on the right side of the lesion) compared to control IgG antibody treated animals. The anti-Nogo-A antibody treated animals had higher scores in sensory-motor tests and showed improved recovery of independent bladder voiding, locomotion and motor coordination.- **From Ref. (Liebscher et al., 2005).**

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2.5 Aims of the thesis

Bladder function after spinal cord injury, the main focus of this thesis, is greatly altered in comparison to healthy subjects. In the long term this represents a life-threatening condition. Currently, there are only symptomatic treatments available and underlying changes in the neuronal circuitry are not fully understood. Causal treatment options are urgently needed.

The first aim of this thesis was to develop a translational animal model to study lower urinary tract function in fully awake animals over time. Current animal models use mostly urethane or other anesthesia to put the animal asleep during measurements, in particular for measurements including the external urethral sphincter EMG. However, it is well known from humans that any kind of anesthesia alters bladder function, what limits the translational value of these measurements. Therefore, it was the first part of the thesis to develop a urodynamic model allowing for repeated measurements in the same animal in the fully awake state including assessment of the external urethral sphincter EMG. In chapter 3 we assessed the new developed urodynamic model and compared it to current standard models using urethane anesthesia.

The second aim of this thesis was to investigate whether our urodynamic model reflects physiological bladder function. This topic raised during the work on chapter 3, where we observed histological alteration after chronic catheter implantation in to the bladder. It was not clear to what extent these changes may influence normal bladder function. We therefore studied in chapter 4 the normal bladder function in naïve rats using metabolic cage measurements and compared it to urodynamic measurements of the same animals.

The third aim of this thesis was first the investigation of the normal course of disease after spinal cord injury to be followed by the assessment of the anti-Nogo-A antibody therapy as first potential causal treatment option. In any treatment trial, it is key to have good understanding of the timeline and the normal course of disease. We therefore investigated in the first part of chapter 5 the development of lower urinary tract

dysfunction, in particular detrusor sphincter dyssynergia, following complete or incomplete spinal cord injury over four weeks in the same animals. With this understanding a first treatment trial with the nerve growth and plasticity promoting anti-Nogo-A antibody was then conducted in a control antibody therapy controlled trial (second part chapter 5).

3 A novel urodynamic model for lower urinary tract assessment in awake rats

by **Marc P. Schneider*** and Francis M. Hughes, Jr.*

* These authors share the first authorship

Further contributions by:

Anne K. Engmann, J. Todd Purves, Hansjörg Kasper, Marco Tedaldi, Laura S. Spruill, Miriam Gullo, Martin E. Schwab and Thomas M. Kessler

M.P.S.: Concieved, initiated and designed the study (with help of F.M.H, A.E., M.E.S and T.M.K). Built the setup and programmed the urodynamics acquisition and analysis software (with help of H.K and M.T.). Performed surgeries. Performed and analyzed urodynamics. Established the digital workflows and extracted all data. Produced all figures (except pictures of Figure 4, they are from L.S.S.). Wrote the manuscript (for methods: with F.M.H., editing by F.M.H., A.E., J.T.P, L.S.S., M.G., M.E.S and T.M.K).

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* MPS and HFM share the first author ship

3.1 Abstract

Objectives: To develop a urodynamic model incorporating external urethral sphincter (EUS) electromyography (EMG) in awake rats.

Materials and methods: Bladder catheters and EUS EMG electrodes were implanted in female Sprague Dawley rats. Assessments were performed in awake, lightly restrained animals on postoperative day 12-14. Measurements were repeated in the same animal on day 16 under urethane anesthesia. Urodynamics and EUS EMG were performed simultaneously. In addition, serum creatinine and bladder histology was assessed.

Results: No significant differences in urodynamic parameters were found between bladder catheter only versus bladder catheter and EUS EMG electrode groups. Urethane anesthesia evoked prominent changes in both urodynamic parameters and EUS EMG. Serum creatinine was within the normal limits in all animals. Bladder weight and bladder wall thickness were significantly increased in both the bladder catheter only and the bladder catheter and EUS EMG group compared to controls.

Conclusions: Our novel urodynamic model allows repetitive measurements of both bladder and EUS function at different time points in the same animal under fully awake conditions and opens promising avenues to investigate LUTD in a translational approach.

3.2 Introduction

Lower urinary tract dysfunction (LUTD) is very common in neurological patients. It affects the lives of millions of people worldwide, has a major impact on quality of life and imposes a substantial economic burden for every health care system (Pannek et al.). Particularly disastrous is detrusor sphincter dyssynergia where neuronal dyscoordination causes the detrusor to contract while preventing sphincter relaxation, resulting in dangerously high spikes in bladder pressure that may lead to kidney damage in the chronic state. Accurate diagnosis of detrusor sphincter dyssynergia requires measurement of the function of both the detrusor and the external urethral sphincter (EUS). Critical to the development of new therapies to combat detrusor sphincter dyssynergia and other LUTD are rodent models that accurately measure both parameters. Unfortunately, current models either lack EUS assessments or utilize anesthesia that is likely to severely alter bladder function. Thus, we aimed to develop and establish an assessment protocol of lower urinary tract function in a rodent that incorporates the synchronous measurement of detrusor activity and EUS function in awake rats, in close analogy to the urodynamic assessment used clinically in humans.

3.3 Materials and methods

Animals (details in Supplement 1): Age-matched female Sprague Dawley rats (260-300 g, 5 mts, Harlan, Frederick, MD, USA) were used in all studies. All experiments were approved by the Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina (USA).

Experimental design (details in Supplement 1): Animals were divided randomly into 3 groups: 1) bladder catheter only group (n=4), 2) bladder catheter and EUS EMG group (n=6), and 3) control (i.e. naïve) group (n=4). Controls were used for creatinine assessment and histology only. To minimize implant-associated bladder dysfunction, urodynamics were not performed immediately but on postoperative day 12-14 on all groups with simultaneous EUS EMG measurement (where appropriate) (Andersson et al., 2011). On day 16 the same rats were administered 600 mg/kg urethane and urodynamics/EUS EMG assessed 30 min later.

Surgery (details in Supplements 1): Animals were anesthetized with ketamine/xylazine and bladder catheters inserted into the bladder dome and secured with a purse string suture. Where indicated, EMG electrodes were affixed to the fat tissue beside the EUS and a ground wire sutured to the abdominal muscle. The bladder catheter and wires were tunneled subcutaneously to the back of the neck and the rat fitted with an infusion harness (QC Single, SAI Infusion Technologies, USA) and allowed 12-14 days to recover.

Urodynamic and EUS EMG measurements (details in Supplement 1): As illustrated in Figure 1a and pictured in Figure 1b, awake animals were positioned in a modified restrainer (modified from item # HLD-RM, Kent Scientific, Connecticut, USA) with a funnel situated under the urethra, as previously described (Hughes et al., 2014). The restrainer was then placed in a modified Small Animal Cystometry Lab Station (Catamount Research and Development Inc.; St. Albans, Vermont, USA) with a scale below the funnel. The bladder catheter was attached to a syringe pump with an in-line pressure transducer and the electrodes (where relevant) connected to an

amplifier/converter. Saline was instilled (120 µL/min) and all parameters (pressure, scale, voltage) recorded simultaneously for at least 3 micturition cycles.

Post-mortem assessments (details in Supplement 1): At sacrifice, blood was obtained by heart puncture and creatinine assessed by standard Enzyme-Linked Immuno-Sorbent Assay (ELISA) techniques. Bladders were removed, weighed and the central third fixed, embedded and sectioned (5 µm). Sections were then stained with hematoxylin and eosin (H&E) or Masson's trichrome stain using routine methodological techniques.

Statistical analysis (details in Supplement 1): Data are reported as mean ± standard deviation (SD). Comparing related and unrelated samples, the paired and unpaired t test was used. To test for differences among the 3 groups, one-way analysis of variance (ANOVA) was applied. The value of significance was considered at $p<0.05$. Statistical analyses were performed using GraphPad Prism, version 6.01 (GraphPad Software, CA, USA).

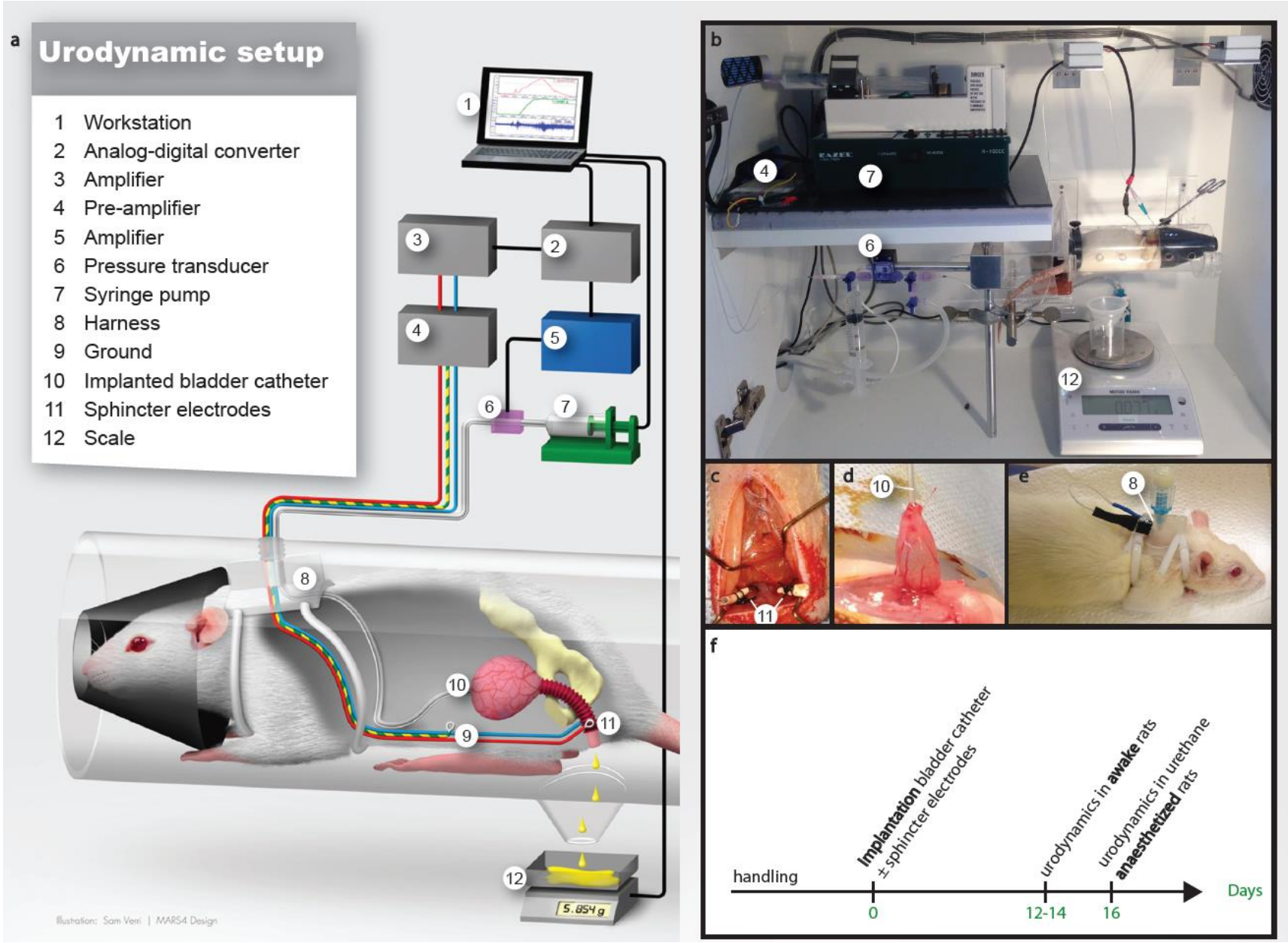

a
Urodynamic setup
1 Workstation
2 Analog-digital converter
3 Amplifier
4 Pre-amplifier
5 Amplifier
6 Pressure transducer
7 Syringe pump
8 Harness
9 Ground
10 Implanted bladder catheter
11 Sphincter electrodes
12 Scale
5.854 g
Illustration: Sam Verri | MARS4 Design
b
c
d
e
f
handling
Implantation bladder catheter
± sphincter electrodes
urodynamics in awake rats
urodynamics in urethane anaesthetized rats
0
12-14
16
Days

Figure 1: a) Scheme of the urodynamic setup. **b)** Urodynamic lab station. **c)** Intraoperative view of the urethra after bilateral implantation of the external urethral sphincter electromyography electrodes. **d)** Intraoperative view of the bladder dome after implantation of the bladder catheter. **e)** Rat with harness affixed. **f)** Study timeline. Numbers in **b-e** relate to the legend in **a.**

3.4 Results

Urodynamic investigation in awake rats: Rats tolerated the harness with the catheter port and the electrode plug very well; no losses (total n=10) were observed over the 3 weeks of the experiment. The animals were acclimated to the urodynamic measurement cabinet for 5 days, after which they stayed in the restraint position during the 1 hr measurement period without any signs of stress or discomfort. A typical analysis from a postoperative day 12-14 rat with bladder catheter and EUS EMG is depicted in Figure 2a and includes a pressure tracing from the bladder, the determination of secreted urine (gr on scale) and the EUS EMG traces. An expanded graph of a single micturition is shown in Figure 2b. Micturition consists typically of four phases (Andersson et al., 2011; Streng et al., 2004) which are indicated on the figure. Phase α: initial increase of intravesical pressure with parallel increase of the EUS EMG activity due to the guarding reflex. Phase β: intravesical pressure increase with high frequency oscillations (pulsatile flow of urine). The EUS EMG shows the specific slow wave bursting. Phase γ: rebound increase in intravesical pressure (end of pulsatile flow). The reappearance of the high amplitude high frequency bursting in the EUS EMG is indicative of a contraction and reappearance of the guarding reflex. Phase δ: rapid intravesical pressure decline to the level before the micturition contraction.

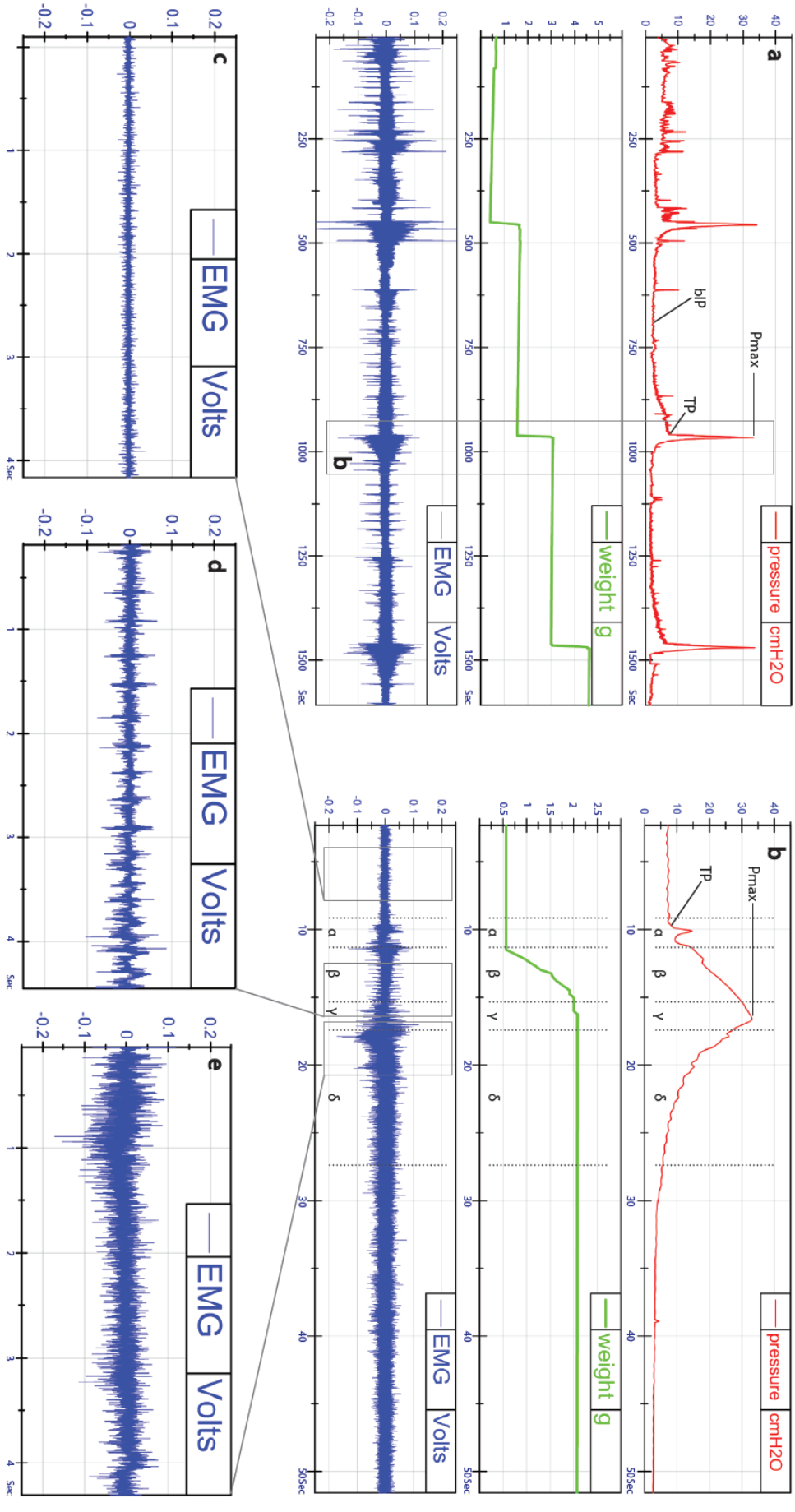

a
pressure cmH2O
weight g
EMG Volts
Pmax
blP
TP
b
c
d
e
Sec

Figure 2: a) 1625 second window of a representative urodynamic tracing from a rat with bladder catheter and external urethral sphincter (EUS) electromyography (EMG) showing three micturition cycles. The first micturition cycle includes moving artifacts and serves for adaptation of the animal. The second and third micturition cycles are representative for an awake rat regardless of group. The top panel shows the bladder pressure tracing, the middle panel the secreted urine weight tracing and the bottom panel the EUS EMG tracing. **bP:** baseline pressure: lowest pressure between two micturitions; **tP:** threshold pressure: pressure shortly before the micturition is started; **Pmax**: maximum voiding pressure: highest pressure during the micturition cycle. **b)** 50 second window culled from **a**. Top panel is the pressure tracing, middle panel the scale tracing and the bottom panel the EUS EMG tracing. The micturition consists of four phases (adapted from [3, 4]): Phase α: initial increase of intravesical pressure with parallel increase of the EUS EMG activity due to the guarding reflex. Phase β: intravesical pressure increased with high frequency oscillations during pulsatile flow of urine. The EUS EMG shows the specific slow wave bursting. Phase γ: rebound increase in intravesical pressure (end of pulsatile flow). The reappearance of the high amplitude high frequency bursting in the EUS EMG is indicative of a contraction and reappearance of the guarding reflex. Phase δ: rapid intravesical pressure decline to the level before the micturition contraction. **c)** 4 second zoomed window from the EUS EMG from **b)** before the micturition has started. Most prominent pattern is a low amplitude high frequency bursting. **d)** 4 second zoomed window from the EUS EMG from **b)** during the micturition. Most prominent pattern is a high amplitude low frequency bursting with medium amplitude high frequency bursting between the slow wave bursting. **e)** 4 second zoomed window from the EUS EMG from **b)** after the micturition. Most prominent pattern is a high amplitude high frequency bursting. **Sec**: second.

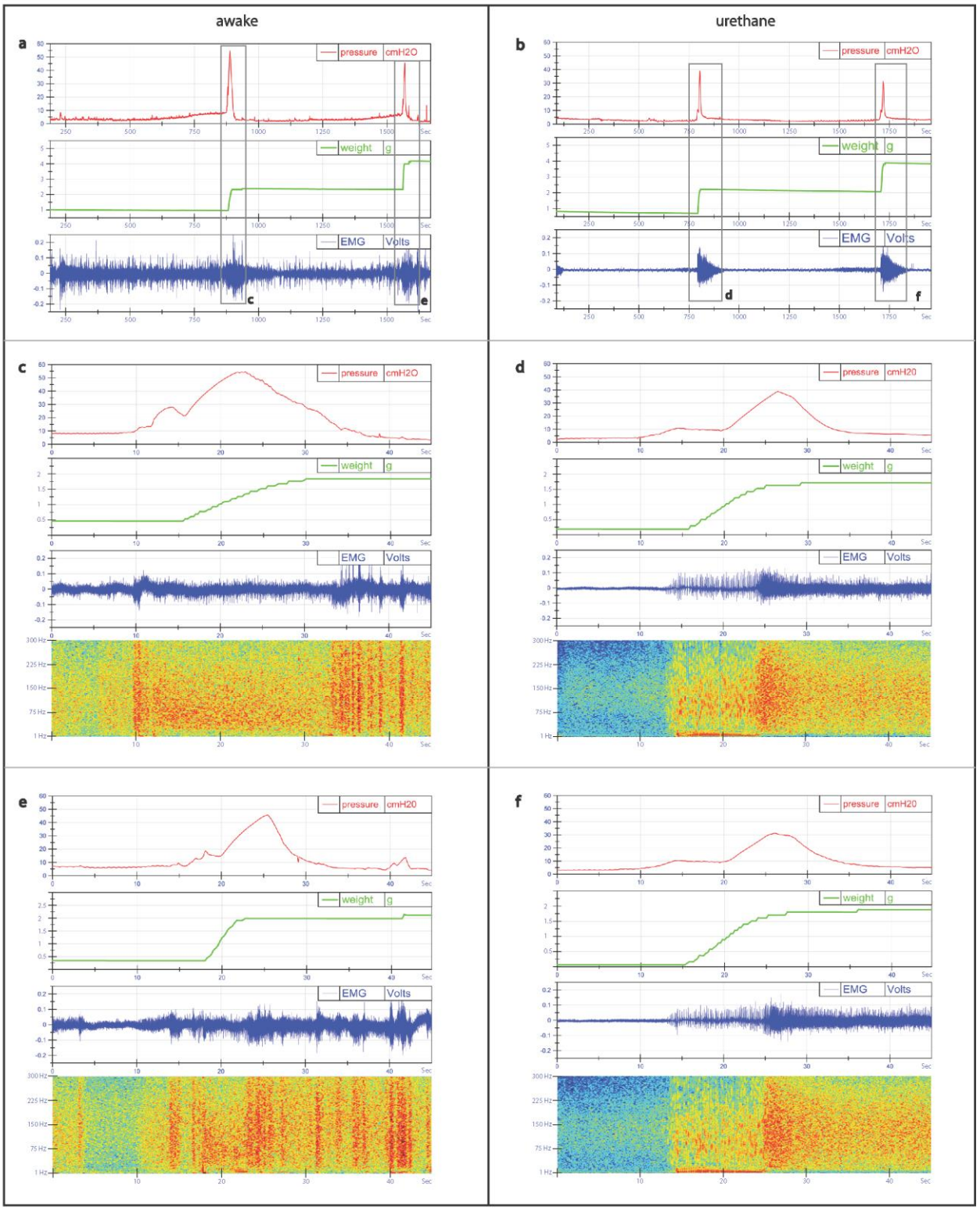
Quantitation of the urodynamic parameters (bladder compliance, mean flow, voiding duration, maximum voiding pressure and voided volume) in the rats with bladder catheter only and rats with bladder catheter and EUS EMG electrodes are presented in Table 1 and demonstrate that there are no significant differences between the two groups.

Table 1. Urodynamic parameters in the bladder catheter only versus the bladder catheter and external urethral spincter (EUS) electromyography (EMG) group

	Bladder catheter group	Bladder catheter and EUS EMG group	p value
Bladder compliance [mL/cmH_2O]	0.27 ± 0.07	0.21 ± 0.09	0.3
Mean flow [µL/sec]	259.0 ± 59.2	251.3 ± 74.63	0.9
Voiding duration [sec]	5.97 ± 0.21	6.71 ± 1.53	0.4
Maximum voiding pressure [cmH_2O]	38.90 ± 13.44	42.19 ± 11.65	0.7
Voided volume [mL]	1.58 ± 0.42	1.63 ± 0.31	0.9

Urodynamic investigation: awake versus urethane anesthetized rats: To assess the effect of urethane anesthesia and to compare our findings in Figure 2/Table 1 to previous studies, all animals (n=10) were administered urethane on post-operative day 16 and urodynamics (± EUS EMG where relevant) assessed 30 min later. Animals from both groups were included in the analysis. Of the 10 animals, 2 had to be excluded: one bladder catheter and EUS EMG electrodes implanted animal died immediately after urethane administration and another (bladder catheter only) was excluded due to dripping overflow incontinence following urethane injection. As shown in Figure 3, urodynamic parameters were significantly altered between awake and urethane anesthetized rats. Anesthesia provoked a decrease in maximum voiding pressure (Figure 3h; p=0.008) as well as an increase in compliance (Figure 3g; p=0.04) and voided volume (Figure 3i; p=0.03). Mean flow rate (p=0.6) and voiding duration (p=0.15) were similar between both groups (data not shown).

EUS EMG parameters were also altered following urethane administration. (Figure 3, n=5). A high frequency pre-micturition burst, similar to the post-micturition burst, was prominent in awake animals (Fig. 3a,c) but highly reduced (in 2 of 5) or not detectable (in 3 of 5) in urethane anesthetized rats (Fig. 3b,d). In addition, baseline amplitude of fast frequency bursting before and during the micturition was reduced in the anesthetized animals. During micturition of urethane anesthetized animals, high frequency bursting activity was almost absent in the intervals between slow wave bursting (Figure 3d).


awake
urethane
a
b
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pressure cmH2O
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EMG Volts
pressure cmH20
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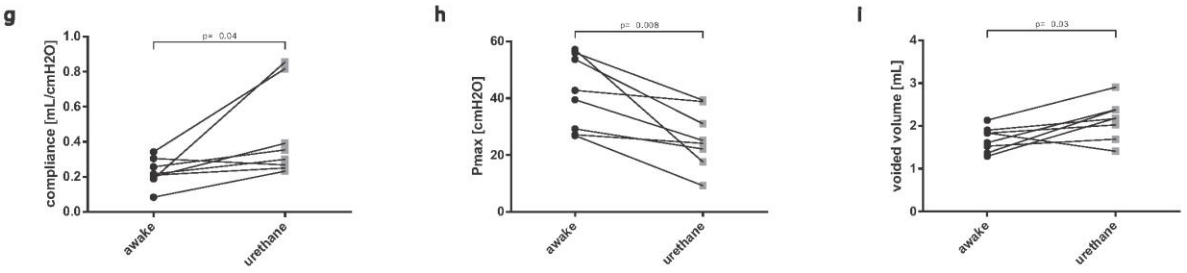
g
compliance [mL/cmH2O]
p= 0.04
awake
urethane
h
Pmax [cmH2O]
p= 0.008
awake
urethane
i
voided volume [mL]
p= 0.03
awake
urethane

Figure 3: **a)** 1500 second window of **a** representative urodynamic tracing with two micturition cycles (**c** and **e**) in an awake rat. Top panel is the pressure tracing, middle panel the scale tracing showing the secreted urine and the bottom panel the external urethral sphincter (EUS) electromyography (EMG) tracing **b)** 1875 second window of a representative urodynamic tracing with two micturition cycles (**d** and **f**) of the same but urethane anesthetized rat. Top panel is the pressure tracing, middle panel the scale tracing and the bottom panel the EUS EMG tracing. **c/e)** 45 second zoomed window from **a** showing urodynamic tracings with time matched frequency spectrograms (bottom panel) of the EUS EMG tracing. Red stands for high amplitude of the specific frequency at this time point, deep blue for low amplitude. Shortly before micturition a band of 4-12 Hz burst simultaneous with a second band of 30-300 Hz bursting is most prominent. During the micturition the 30-300 Hz bursting is less prominent (in 5 out of 5 animals). At the end of micturition the 4-12 Hz slow bursting disappears and the 30-300 Hz bursting gets very prominent for 5-10 seconds. **d/f)** 45 second zoomed window from **b** showing urodynamic tracings with time matched frequency spectrograms (bottom panel) of the EUS EMG tracing. Red stands for high amplitude of the specific frequency at this time point, deep blue for low amplitude. Before micturition there is only very little bursting in any frequency. During the micturition the 4-12 Hz slow wave bursting is very prominent (in 5 out of 5 animals). At the end of micturition the 4-12 Hz slow bursting disappears and the 30-300 Hz bursting gets very prominent for 5-10 seconds. **g)** Bladder compliance of the individual animals in the awake compared to the urethane anesthetized state (n=8, p=0.04). **h)** Maximum voiding pressure (Pmax) of the individual animals in the awake compared to the urethane anesthetized state (n=8, p=0.008). **i)** Voided volume of the individual animals in the awake compared to the urethane anesthetized state (n=8, p=0.03).

Post-mortem analysis: As shown in Figure 4a, serum creatinine levels in the experimental, implanted animals were within the normal range (<88 μmol/L) with no significant differences to the controls. However, bladder weight and bladder wall thickness were increased more than two-fold in both the bladder catheter only and the bladder catheter and EUS EMG group compared to controls (Figure 4b and 4c). These same groups displayed marked muscular hypertrophy and urothelial hyperplasia (Figure 4d-f). Masson's trichrome staining for collagen was similar in all 3 groups (Figure 4g-i) and there were no signs of bacterial infection.

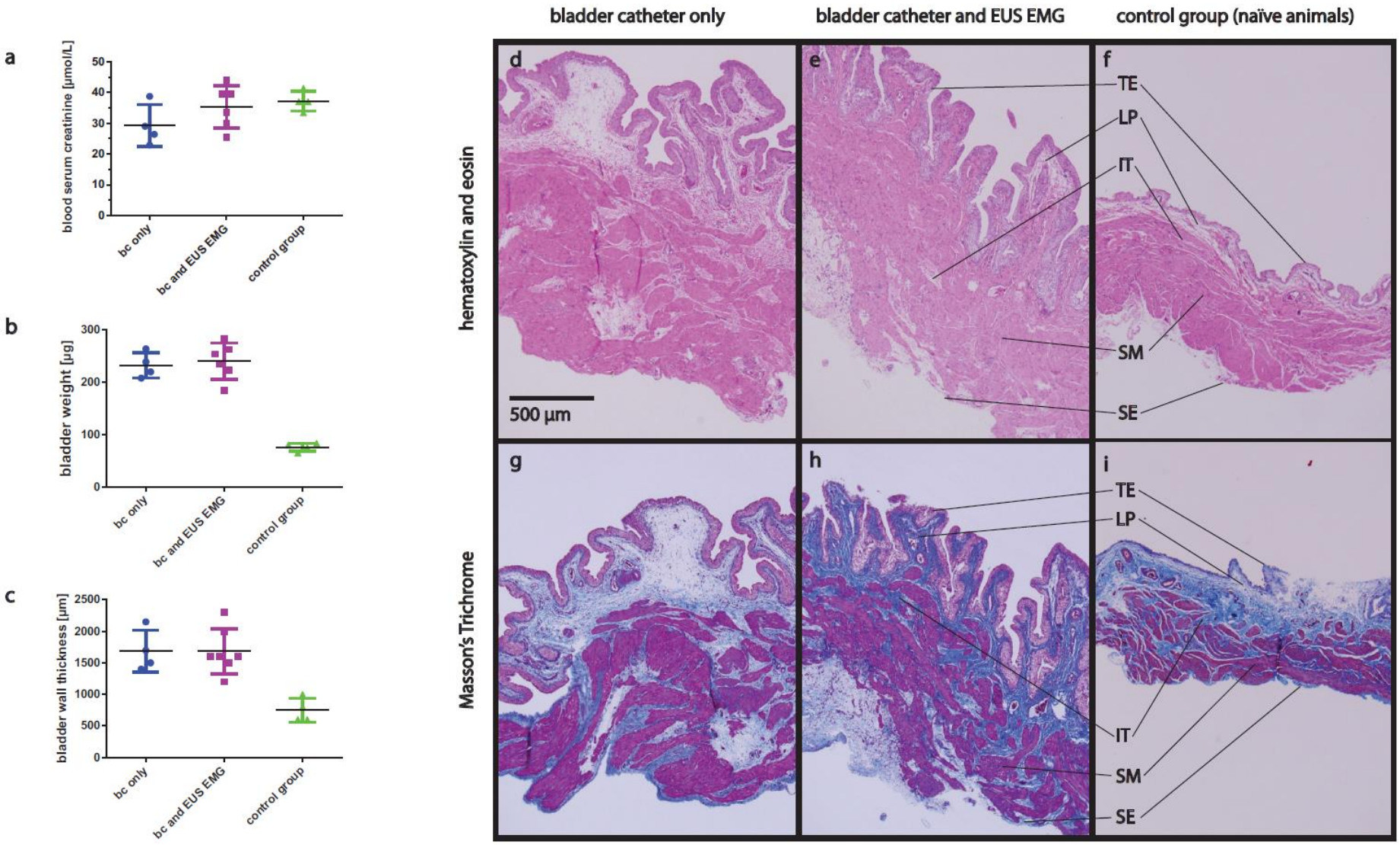
a
blood serum creatinine [µmol/L]
50
40
30
20
10
0
bc only
bc and EUS EMG
control group
b
bladder weight [µg]
300
200
100
0
bc only
bc and EUS EMG
control group
c
bladder wall thickness [µm]
2500
2000
1500
1000
500
0
bc only
bc and EUS EMG
control group
bladder catheter only
bladder catheter and EUS EMG
control group (naïve animals)
hematoxylin and eosin
Masson's Trichrome
d
e
f
g
h
i
500 µm
TE
LP
IT
SM
SE
TE
LP
IT
SM
SE

Figure 4: a) Blood serum creatinine levels in rats with bladder catheter only **(bc only)**, combined bladder catheter and EUS EMG electrodes **(bc and EUS EMG), or** in control (naïve) animals **(control group)**. **b)** Bladder weights of the same groups depicted in **a**. **c)** Bladder wall thickness of the same groups depicted in **a**.**d/e/f)** Histological sections of bladders obtained from the same groups depicted in **a** and stained with H&E demonstrating muscular hypertrophy, urothelial hyperplasia and increased edema between the mucosal layer and the detrusor in the experimental groups as compared to the controls. **g/h/i)** Histological sections of bladders dissected from the same groups depicted in **a** and stained with Masson's trichrome demonstrating a proportional increase in collagen without increased fibrosis in the experimental groups as compared to the controls.**TE**: Transitional epithelium; **LP**: Lamina propria; **IT**: Interstitial connective tissue; **SM**: Smoth muscle bundles; **SE**: Serosa.

3.5 Discussion

Our findings demonstrate that chronic, combined bladder catheter and EUS EMG electrodes in the same animal do not impair bladder function in the awake rat. On the other hand, urethane anesthesia significantly alters both detrusor and EUS activities. To the best of our knowledge, this is the first presentation of a rodent urodynamic model for repetitive lower urinary tract assessment that includes EUS EMG analysis in an awake animal. Moreover, given the nondestructive nature of the measurements, this model allows for repetitive analysis at different time points in the same animal. Thus, our novel urodynamic rodent model opens promising avenues to investigate LUTD in a translational approach.

Anesthetic drugs are well known to impair lower urinary tract function (Wuethrich et al., 2011; Wuethrich, Kessler, et al., 2013). Thus, to represent the situation in everyday life as close as possible, human urodynamics (which includes EUS EMG) is performed in an awake state without anesthetics (Gammie et al., 2014). In animals, however, all existing studies which included urodynamics and EUS EMG were carried out under anesthesia (D'Amico et al., 2011; Kakizaki et al., 1997; Kruse et al., 1993). Although urethane seems to be the best available anesthetic to maintain the micturition response (Andersson et al., 2011; Matsuura & Downie, 2000), it strongly impairs bladder function, leading to significant differences in urodynamic findings compared to the awake state (Aizawa et al., 2015). In the present study, we observed lower baseline amplitude of high frequency bursting before, during and after micturition in the urethane-treated rat, showing the lower basal EUS activity. Decreased EUS activity results in lower bladder outlet resistance which might explain the lower maximum voiding pressure in the anaesthetized animals since less pressure is needed to overcome a lower infravesical resistance.

It is described in literature, basing on urethane anaesthetized measurements, that the slow wave bursting, the most prominent pattern during voiding, facilitates a sufficient urination (Kruse et al., 1993). Leung et al. (Leung et al., 2007) generally supported this

opinion in a series of experiments using restrained, awake animals tested shortly after the implantation of the bladder catheter and EUS EMG electrodes. However, their model is hampered by the fact that measurements were performed immediately after surgery where postoperative pain and the anesthetics used for the implantation surgery are likely to have affected bladder function. Additional, as mentioned by Andersson et al. (Andersson et al., 2011), the implantation causes acutely smaller voiding volumes that corresponds with a frequency symptomatic that normalizes after some days. In contrast, LaPallo et al. (LaPallo et al., 2014) assessed EUS EMG activity over time in unrestrained awake rats and did not detect EUS slow wave bursting activity during voiding in about 25% of the animals. Correlation of those studies with the present one is difficult since LaPallo et al. (LaPallo et al., 2014) did not assess bladder function with simultaneous intravesical pressure measurement. It is possible that the 25% of animals that did not display slow wave bursting were suffering from a LUTD. Moreover, there were significant differences in the electrode implantation techniques used in our study versus that of LaPallo et al. (LaPallo et al., 2014). In LaPallo's study (LaPallo et al., 2014), the EUS EMG electrodes were affixed intra-abdominally to the pelvic bone, whereas in the present study we have used an extra-abdominal pelvic approach and affixed the electrodes to the fat tissue beside the EUS (Figure 1c and Supplement 2). These alternative approaches may contribute to the differences between the two studies.

Urethane is described by Hara et al. (Hara & Harris, 2002) as having no single predominant target channel but rather affecting multiple channels simultaneously, suggesting that neurotransmitter systems in the central nervous system might also be affected. Thus, careful use of urethane as an anesthetic for any neurophysiological measurements is highly warranted.

The pre-micturition high frequency burst detected in our awake animals was almost identical to the post-micturition burst. Interestingly, Kakizaki et al. (Kakizaki et al., 1997) also observed similar high frequency bursting following induced reflex bladder contractions. One possible explanation for this phenomenon is that the pre-micturition

burst might be due to an EUS contraction induced by the guarding reflex just before micturition begins. Under urethane anesthesia this pre-micturition burst disappeared in our study, similar to other reports in the literature (Cruz & Downie, 2005; Kruse et al., 1993). This result highlights the significant influence urethane exerts on lower urinary tract function.

One major issue in urodynamics in rats is the high inter-animal variability. Since urodynamic assessment under urethane anesthesia necessitates sacrifice after investigation, large numbers of animals are needed per group to detect significant differences. Our novel urodynamic model allows for repetitive measurements at different time points in the same awake animal. Testing an animal before and after treatment allows that animal to serve as its own control and allows assessment relative to that animal's individual baseline. This eliminates the problems associated with inter-animal variability and dramatically reduces the number of animals needed to detect significant changes, ultimately reducing experimental time, costs, and resources without compromising statistical quality.

The evidence is clear that anesthetics affect bladder function, as shown by others (Wuethrich et al., 2011; Wuethrich, Kessler, et al., 2013; Wuethrich, Metzger, et al., 2013) and the present study. Consequently, animal models that utilize anesthetics are problematic and the translational value of the findings is questionable. In line with the International Continence Society Guidelines on Urodynamic Equipment Performance in humans (Gammie et al., 2014), it is suggested that all urodynamic assessments in animal models be performed in an awake state to avoid major bias by narcotics.

A high pressure system puts at risk the upper urinary tract. In humans, intravesical pressures that spike to >40 cmH_2O during the storage phase are generally agreed to jeopardize renal function so that an appropriate treatment is needed (McGuire et al., 1981). Thus, the high spikes in pressure caused by detrusor overactivity and detrusor sphincter dyssynergia can cause significant kidney damage and accurate diagnosis in humans requires measurement of both detrusor and urethral sphincter function (Blok

et al., 2016). Our model allows for simultaneous detrusor and EUS assessment in awake rats for the first time and thus promises to be a very useful tool for future translational research on detrusor overactivity and detrusor sphincter dyssynergia specifically and LUTD in general. The absence of urethane narcosis is critical for these future studies as anesthesia dampens pressure spikes. The risk that detrusor overactivity / detrusor sphincter dyssynergia are not recognized under urethane anesthesia is high and the effectiveness of a tested treatment may be underestimated.

The main limitation of our study is the small number of animals investigated. However, our findings are well in line with the literature and our model combines for the first time bladder and EUS assessment in awake animals. Another limitation is that histology showed urothelial hyperplasia and detrusor hypertrophy in both the bladder catheter only as well as the combined bladder catheter and EUS EMG electrode implanted rats. There was no increase in collagen content, however, suggesting that bladder catheter implantation did not cause bladder fibrosis. The implantation-induced tissue alterations need to be considered when bladder specific processes are assessed. In humans, combined pelvic floor EMG and videocystourethrography (VCUG) during urodynamic investigation are the most acceptable and widely agreed methods for diagnosis of DSD (Suzuki Bellucci et al., 2012), especially considering that both detrusor internal and external spincter dyssynergia can be investigated. VCUG is not yet available in rats but we are working on some additional improvements and in the optimal case a video-urodynamic assessment could be established. Thus, detrusor internal sphincter dyssynergia (bladder neck dyssynergia) is currently not evaluated in our animal model. So far, EUS EMG signals were only analyzed semi-quantitative, this is according to urodynamic investigations in humans. However, software for quantitative assessments is under development.

In conclusion, our novel urodynamic model allows repetitive measurements of both bladder and EUS function at different time points in the same animal under fully awake conditions, opens promising avenues to investigate LUTD in a translational approach. In future studies, we will use this model to investigate major neurological diseases

causing LUTD such as spinal cord injury (Liebscher et al., 2005), multiple sclerosis (Hiroya Mizusawa et al., 2000) and stroke (Wahl et al., 2014) where we expect it to provide better understanding of the underlying mechanisms involved. In addition, our model can be used to assess new causal therapeutic options for these diseases.

3.6 Supplement

Animals

All animals were age-matched female Sprague Dawley rats (264-312g, Harlan, Frederick, MD, USA). Upon arrival animals were housed in an Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC)-approved colony room in groups of 2 animals per cage at a 12:12 light-dark cycle with food and water provided ad libitum. Rats were given at least seven days to acclimate to the facility before experiments began. After surgery animals were housed singly. All experiments were performed in accordance with the guidelines set forth in the National Institutes of Health (NIH) Guide for the Care and Use of Laboratory Animals, published by the Public Health Service of the United States of America (USA), and were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) at the Medical University of South Carolina.

Animals were divided randomly into 3 groups: 1) bladder catheter only group (n=4), 2) combined bladder catheter and external urethral sphincter (EUS) electromyography (EMG) group (n=6), and 3) control group (n=4) without any catheters, i.e. naïve group. Of the 10 animals 2 had to be excluded: one combined bladder catheter and EUS EMG electrodes implanted animal died immediately after intraperitoneal injection of the urethane and another (bladder catheter only group) was excluded due to dripping overflow incontinence directly after administration of the urethane.

Bladder catheter and EUS EMG electrode preparation

Catheters were prepared from 24 cm sections of PE-50 tubing (Polyethylene, Scientific Commodities, Inc., Lake Havasu City, Arizona, USA) by flaring one end on a heating surface. A 3 mm section of silicon tubing (type 00702, diameter 0.7/1.1mm; Detakta, 22851 Norderstedt, Germany) was advanced from the opposite end to come to rest underneath the flare. This piece of tubing served as a spacer separating the luminal

opening from the urothelium, thus preventing the growth of the urothelium over the flare, resulting in occlusion. Tubing was then sterilized by ethylene oxide exposure.

EMG electrodes (Figure 1.a.11, round tip; 1 mm in diameter) were prepared from silver wire (AGI1025, World Precision Instruments, Inc., FL, USA) soldered to Teflon-coated steel wire (Cooner Wire, 9265 Owensmouth Avenue, Chatsworth, California 91311, USA). To avoid electrochemical corrosion of the steel wire the electrode/wire connection was coated with silicone (3140 TTV Coating, Dow Corning Corporation, Midland, Michigan, USA) and sealed with a thin, 5 mm long, piece of shrink tubing diameter 0.86mm Type TFE2-30-1220-PTFE-CL (Hellermann Tyton, Austria). Electrodes were then sterilized by ethylene oxide exposure.

Bladder catheter and EUS EMG electrode implantation

To prepare rats for urodynamic investigation, catheters were implanted in the dome of the bladder while EMG electrodes were sutured on either side of the EUS to the lateral fat tissue (Figure 1.c.11). The approximate locations of these are illustrated in Figure 1.a. Under ketamine (90mg/kg) and xylazine (10mg/kg) anesthesia, the abdominal cavity was opened ventrally, the bladder exposed and a small incision made in the bladder dome. The flared end of the catheter was inserted into the bladder and fixed with a purse string suture using 6-0 prolene sutures equipped with a tapered non-cutting needle (Figure 1.d.10). The catheter was then tunneled subcutaneously and exteriorized at the back of the neck and secured in place with sutures (5-0 silk) into the interscapular muscle. The muscle layers of the abdominal cavity were closed with 5-0 polyglycolic acid (PGA) sutures. Animals in the bladder catheter only group had their skin layer sutured with the same material.

For rats in the combined bladder catheter and EUS EMG group the urethra was localized and a pocket prepared by blunt dissection up to the pelvic floor muscles on each side. Separate silver tipped electrodes were fixed to the fat tissue on either side of the EUS with several stiches (5-0 silk) (Figure 1.c/Supplement 2). No stich was set directly into the EUS or urethra. Fixation to the fat tissue was sufficient since the

tension of the tissue after closing of the incision pushed the electrodes slightly towards the EUS and urethra and kept them in place. A third identical electrode was used as ground and sutured (5-0 silk) to the abdominal muscles approx. 1.5 centimeter rostral to the EUS electrodes (Figure 1.a.9). All three wires were tunneled together subcutaneously to the neck. The fat tissue of the abdominal incision was closed separately (5-0 PGA) above the electrodes as cushion to avoid necrosis and penetration. The skin was then closed with 5-0 PGA.

Both bladder catheter only as well as combined bladder catheter and EUS EMG electrode animals were fitted with an infusion harness (SAI QC Single, SAI Infusion Technologies, USA)(Figure 1.a.8) and the catheter plugged with a 23g plug, which was inserted into the harness. The wires extending from the combined bladder catheter and EUS EMG electrode animals were soldered into a connector (Preci-dip, Serie310, Digikey 1212-1105-ND) which was affixed to the harness using a fast drying cyanoacrylate glue (for example Loctite 4500). Animals remained in these harnesses through the course of the experiment.

Immediately after surgery and once per day for the following 6 days, antibiotics (Borgal 24%; trimethoprim and sulfadoxine 1:5; Virbac, France) were injected subcutaneously (15 mg/kg). The analgesic Carprofen (5 mg/kg, Pfizer AG, USA) was also given directly after the operation and then 1-2 times per day for the following two days (frequency depended on the wellbeing and appearance of the animals).

Animal care

Intensive daily handling of the animals was started one week prior to the bladder catheter and EUS EMG electrode implantation. Following implantation animals were checked three times daily. Once a day the emersion point of the bladder catheter and EUS EMG electrodes was disinfected with a standard povidone-iodine solution (i.e. Betadine). To avoid denting and compression necrosis, the position of the harness and the tightness of the rubber bands were checked daily.

Urodynamic investigation

For analysis the animals were placed in a modified restrainer (modified from item # HLD-RM, Kent Scientific, Connecticut, USA) with a small hole near the rear which was fitted with a funnel. The animals were situated in the station so that the funnel sat over an analytical balance (Mettler-toledo, Greifensee, Switzerland) (Figure 1.a.12) that recorded the weight of voided urine, thus providing an index of the voided volume. The bladder catheter was attached to a computer-controlled syringe pump (Figure 1.a.7) through an in-line pressure transducer (Figure 1.a.6) that recorded the intravesical pressure during the infusion. The bladder pressure signal was amplified by the signal conditioner (Figure 1.a.5 Catamount Research and Development Inc., St. Albans, Vermont 05478). In the combined bladder catheter and EUS EMG electrode group, the EMG signal was simultaneously measured. For this the electrode was connected to a differential preamplifier (Figure 1.a.4). From there the signal is passed to the amplifier (Figure 1.a.3) using a bandpass filter (2 Hz to 2 kHz,). Both the preamplifier and amplifier were constructed in-house specifically for this purpose (Brain Research Institute, Zürich, Switzerland). All signals where sampled at 10 kHz using the analog to digital converter (Figure 1.a.2; NI USB-6211, National Instruments, Austin Texas, USA). Control of the entire urodynamic setup, including infusion rate by the syringe pump and all data acquisition were performed with a self-programmed application (Figure 1.a.1) based on LabVIEW v2012 (National Instruments, Austin, Texas, USA).

For urodynamic investigations, the syringe instilled saline into the bladder at a constant rate (120 µL/min). Bladder pressure, EUS EMG activity, infused and voided volume (scale reading) were continuously measured. A micturition cycle was defined as the period from just after one micturition until just after the next. The first micturition cycle was observed to find any problems (e.g. linear increasing pressure indicating an obstructed or a kinked catheter) and used as acclimatization for the animal. This cycle was not counted for analyses. The infusion was continued for 45-90 min until at least three micturition cycles were measured.

Urodynamics on awake animals was performed at the times indicated in Figure 1.f (on day 12 to 14). On day 16, bladder catheter only (n=4) as well as combined bladder catheter and EUS EMG electrode (n=6) rats were administered urethane (intraperitoneal, 600 mg/kg; 50% of the full anesthetic dose; Sigma Aldrich, St. Louis, MO, USA) and urodynamics repeated 30 minutes later. The animals were euthanized immediately after urethane urodynamics. During each urodynamic session, the animal was assessed to determine if it was relaxed in the restrainer after the acclimatization time of approx. 10 minutes, indicated by a normal breathing rate, absence of frequent movements and signs of pain.

For data assessment, the maximum voiding pressure (Pmax) was measured as the peak of the intravesical pressure during voiding (Figure 2.a). Voided volume was measured as the change in weight on the scale (assuming a density approaching 1). The threshold pressure (tP) was defined as the intravesical pressure just prior to the start of micturition. The lowest pressure, usually just after a micturition, was recorded as baseline pressure (bP). To serve as a measurement of the rigidity of the bladder wall, bladder compliance was calculated as the intravesical bladder pressure increase (tP minus bP) divided by the voided volume. The EUS EMG was highpass filtered, full wave-rectified and averaged width a 4Hz IIR zero phase Butterworth lowpass filter which corresponds to a moving average filter with Hanning window. For this DIAdem v2011 (National Instruments) was used.

Blinding during urodynamic data acquisition was not possible due to the obvious effect of the urethane anesthesia. Blinding of the urodynamic readout was performed by a second person (FMH) who encrypted the animals ID and the measuring time points. Unblinding was accomplished after data collection (MPS).

Spectrogram

Analyses were performed with a self-programmed application based on LabVIEW version 2012 (National Instruments, Austin, Texas, USA). The 45 second long EMG signal is sliced into 4096 samples, of whom 3596 are overlapping (shift is 500

samples). After taking a hanning window of the signal, a fast Fourier transformation was generated. As a result we get a power value for each frequency at every computed time point. Before graphic processing, the signal was up scaled by a multiplication factor to assure that the signal consists, after logarithmizing, mainly of positive values. The logarithmized signal was also scaled up by factor 20. This signal was then plotted as color map with Jet Colormap. All frequencies above 300 Hz were neglected.

Analysis of blood serum creatinine

At sacrifice, rats were anesthetized with ketamine (90 mg/kg) and xylazine (10 mg/kg) and blood samples taken by heart puncture. Red blood cells were pelleted (500 x g) and serum creatinine levels measured by the Veterinary Diagnostic Laboratory at the Medical University of South Carolina using standard Enzyme-Linked Immuno-Sorbent Assay (ELISA) techniques.

Histology

After taking of blood samples at sacrifice, correct position of the EUS EMG electrodes was visually confirmed, bladders were removed and catheter and suture dissected from the isolated bladders. The upper third, including the former catheter implantation site, and the lower third of the bladder was dissected away. The resulting central portion of the bladder was immersed overnight in 10% neutral buffered formalin (4°C). Bladders were transferred to 70% ethanol and maintained at 4°C until embedded in paraffin blocks using standard techniques. Sections (5 µm) were cut and stained with hematoxylin and eosin (H&E) and Masson’s trichrome using routine methodological techniques.

A Video of the catheter and electrodes implantation surgery can be downloaded under:

http://onlinelibrary.wiley.com/store/10.1111/bju.13039/asset/supinfo/bju13039-sup-0002-si.MP4?v=1&s=4994cedf4a2d025d9ac6c7a057bee880049d4c8b

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4 Do urodynamic measurements reflect physiological bladder function in rats?

by **Marc P. Schneider**

Further contributions by:

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M.P.S.: Conceived, initiated and designed the study (with help of M.E.S and T.M.K). Built the setup and programmed the metabolic cage acquisition and analysis software. Performed surgeries (with help of S.M.). Performed and analyzed urodynamics and metabolic cage (with help of J.T., A.S. and S.M.). Established the digital workflows and extracted all data (with help of J.T. and S.M.). Produced all figures (with help of J.T. and S.M.). Wrote the manuscript (editing by A.S., J.T., S.M., A.E., B.V.I., A.S.H., M.E.S and T.M.K).

Submitted for publication

4.1 Abstract

Our objective was to investigate and compare bladder function in rats assessed by metabolic cage and by urodynamic measurements in fully awake animals. Bladder function of female Lewis rats was investigated in naïve animals by metabolic cage at baseline, 14-16 days after bladder catheter and external urethral sphincter electromyography electrode implantation in fully awake animals by urodynamics, and again by metabolic cage. Investigating the same animals (n=8), voided volume, average flow and duration of voiding were similar ($p>0.05$) in naïve animals measured by metabolic cage and after catheter implantation by urodynamic measurements and by metabolic cage. In naïve animals measured by metabolic cage, voided volumes were significantly different in the light (resting phase) versus the dark (active phase) part of the 24 hr cycle (mean difference 0.14mL, 21%, $p=0.004$, n=27). Lower urinary tract function assessed by metabolic cage or by urodynamic meaurements in fully awake rats was indistinguishable. Thus, catheter implantation did not significantly change physiological bladder function. This shows that urodynamic measurements in awake animals are an appropriate translational approach to study lower urinary tract function in health and disease in animal models, directly paralleling the human diagnostic procedures.

4.2 Introduction

Urodynamic investigation in fully awake animals represents the most direct translational approach for assessing bladder function in rats (Andersson et al., 2011; Schneider, Hughes, et al., 2015). However, this procedure requires chronic implantation of a bladder catheter causing a local tissue reaction with thickening of the bladder wall (Schneider, Hughes, et al., 2015). This raises the question to what extent the implanted catheter influences bladder function and consecutive urodynamic measurements. Andersson et al. observed that catheter implantation reduced the voided volume during the first days after implantation reaching a stable plateau after three to five days (Andersson et al., 2011). In 2008, Smith et al. demonstrated in catheter implanted animals that most bladder function parameters were highly comparable in the micturitions triggered by repeated bladder filling via a catheter or during spontaneous voiding with naturally filled bladder through renal filtration (Smith et al., 2008). These measurements, however, where performed within a rather short period after catheter implantation and under urethane anesthesia, which is well known to influence and change bladder function (Schneider, Hughes, et al., 2015). Thus, it remains to be elucidated if catheter implantation and/or the bladder filling via a catheter, influences urodynamic measurements in a relevant way, thereby limiting the translational value of all current urodynamic rat models. Here, we compared in the same animals' bladder function measured in naïve animals by metabolic cage at baseline with bladder function in catheter implanted animals assessed by urodynamic measurements and by metabolic cage.

4.3 Materials and methods

Animals: Age-matched female Lewis rats (LEW/OrlRj (Lewis), 210 g ± 20 g, 4 mts ± 1mt, Janvier, France) were used in all studies. The animals were housed in groups of three to four per cage (single housed after catheter implantation), food and water were provided *ad libitum* (standard rat chow and water). Rats were maintained on a 12/12 h light/dark cycle (light on from 6:00 a.m. until 6:00 p.m). Urodynamic measurements were performed during the light phase, while metabolic cage measurements for 24 hours on a 12/12 h light/dark cycle. All experiments were approved by the Veterinary Office of the canton of Zürich, Switzerland (License nr. 19/2014) and were in accordance with the approved guidelines and regulations.

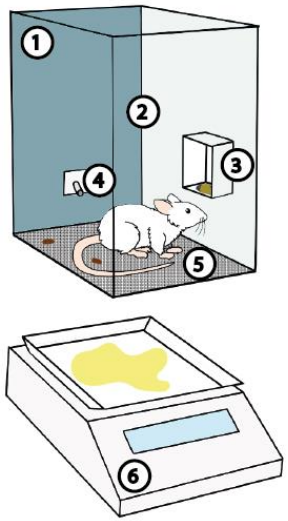
Experimental design (see also Figure 1a, d): All catheter and electrodes implanted animals (n=8) were treated according to the same protocol: after the initial preoperative baseline measurement in the metabolic cage, catheter and electrodes were implanted. To minimize implantation-associated bladder dysfunction, urodynamics with simultaneous external urethral sphincter electromyography (EMG) measurements were not performed immediately but on postoperative day 14-16 (Andersson et al., 2011). Following a one-day break, the same rats were measured again in the metabolic cage. 19 additional animals were only measured initially, in a naïve baseline metabolic cage assessment.

Metabolic cage measurement: Awake animals were positioned in a Small Animal Cystometry Lab Station (Catamount Research and Development Inc.; St. Albans, Vermont, USA) with a modified grid as bottom of the cage, allowing urine to drip onto a scale, whereas the faeces were retained by the grid. The grid of the metabolic cage leads to a delay of the urine dripping on the scale and thereby to a prolonged micturition duration compared to urodynamic measurement where the urine is caught on the scale directly. To avoid measurement bias, we only compared voiding duration and mean flow in metabolic cage naïve animals versus metabolic cage catheter implanted animals where the measurement situation is identical. To correct for the loss of urine

on the grid, a calibration trial was undertaken where different saline volumes (0.2-1.4mL) were pipetted ten times, each volume at the exact same ten places for each different volume (followed by drying of the grid after each run). The mean weight measured on the scale was calculated and used to plot a calibration curve for percental loss per micturition (Figure 1b). A correction formula was generated from a fitted line (r^2 = 0.504). The exponential formula (Figure 1c) was used to correct the weight measured on the scale to the estimated real voided volume. Animals were measured for 24 hours starting at noon and were kept in a 12/12 light dark cycle, switching at 06:00 a.m. and 06:00 p.m., and had access to food and water *ad libitum* during the measurement.

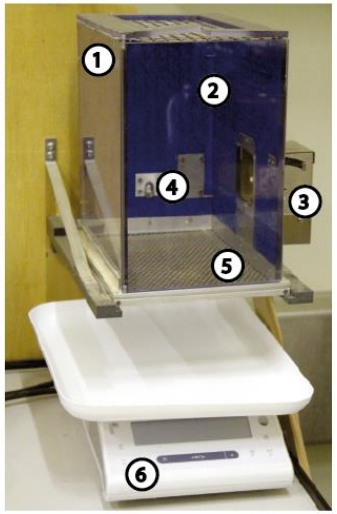
Figure 1

a. Schematic view and picture of metabolic cage setup

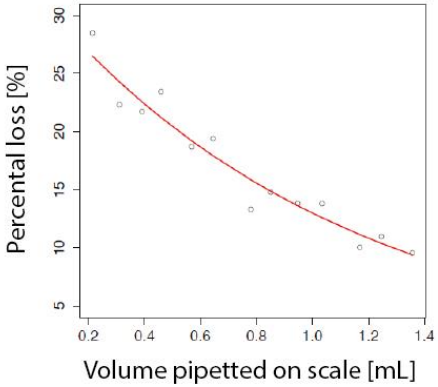


Metabolic cage setup

1. animal cage
2. 2 opaque and 2 transparent walls
3. food tray
4. water supply
5. grid, filtering feaces
6. scale measuring voided urine over time



b. Calibration curve for percental loss per micturition



c. Conversion formula

$$z = x + e^{0.40x - 2.35}$$

z being the original volume
x being the recorded volume

d. Study timeline

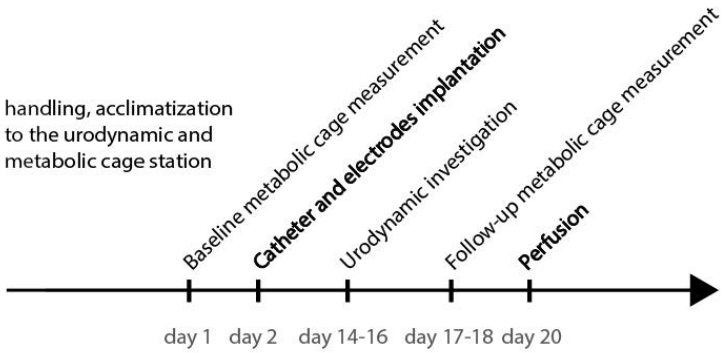


Figure 1: a. Schematic view and picture of metabolic cage setup. **b.** Calibration curve for percental loss per micturition. **c.** Conversion formula; z being the original volume, x being the recorded volume. **d.** Study timeline.

Catheter and electrodes implantation (as described previously (Schneider, Hughes, et al., 2015)): Animals were initially anesthetized in 5% Isoflurane (Piramal Healthcare) in air and maintained by an i.m. injection of Medetomidine (0.15 mg/kg Domitor, Orion Pharma), Midazolanum (2 mg/kg, Dormicum, Roche) and Fentanyl (5 µg/kg, Fentanyl, Kantonsapotheke University Hospital Zürich). Bladder catheters were inserted into the bladder dome and secured with a purse string suture. EMG electrodes were affixed to the fat tissue beside the external urethral sphincter and a ground electrode was sutured to the abdominal muscle. Catheter and wires were tunneled subcutaneously to the back of the neck, exteriorized, and lastly attached to an infusion harness (QC Single, SAI Infusion Technologies, USA).

Urodynamic and external urethral sphincter EMG measurements (as described previously (Schneider, Hughes, et al., 2015)): Acclimatization of the animals to the urodynamic setup was performed before catheter and electrode implantation. Awake animals were positioned in a modified restrainer (modified from item # HLD-RM, Kent Scientific, Connecticut, USA) with a hole situated under the urethra. The restrainer was then placed in a modified Small Animal Cystometry Lab Station (Catamount Research and Development Inc.; St. Albans, Vermont, USA) with a scale below the funnel. The bladder catheter was attached to a syringe pump with an in-line pressure transducer and the electrodes were connected to an amplifier/converter. Saline was instilled (120 µL/min) and all parameters (pressure, scale, voltage) recorded simultaneously for at least 3 micturition cycles.

Statistical analysis: Data are reported as mean ± standard deviation (SD). For comparison of related and unrelated samples, the paired and unpaired t test was used. The value of significance was considered at $p<0.05$. Statistical analyses were performed using STATA statistical software, version 14 (StataCorp, College Station, Texas, USA).

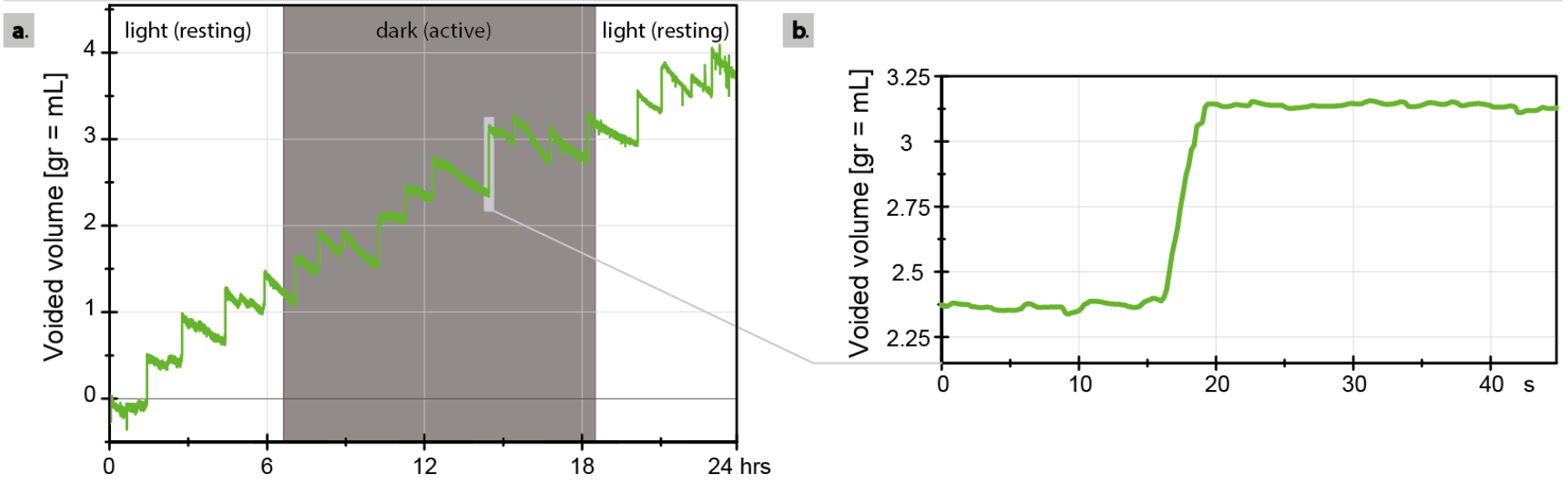
4.4 Results

Metabolic cage measurement: Rats tolerated the metabolic cage measurements without any recognizable distress. A typical sequence of micuritions over 24 hrs from a naïve rat is shown in Figure 2a (the weight of the voided urine decreases by evaporation after each voiding; maximum voided volumes are calculated from the peak values). A magnified trace of a single micturition is shown in Figure 2b. Quantitation of the micturition parameters in the metabolic cage (voided volume, average flow and voiding duration) are presented in Table 1 and Figure 3. Importantly, data show no significant differences between the baseline measurements in the naïve rats and the measurements after catheter and electrode implantation in the metabolic cage. Comparing metabolic cage measurements during light versus dark phase, we found during light phase reduced mean number of micturitions per hour, significant increased voided volumes and reduced average duration of voiding what lead in combination to an increase in average flow during voiding.

Urodynamic investigation: Urodynamic measurements were well tolerated by the animals and no abnormal behavior was observed. In Figure 2c, a typical urodynamic tracing of an entire measurement session is depicted, and Figure 2d shows a zoom of a single micturition. External urethral sphincter EMGs show low-amplitude high-frequency bursting just before voiding initiation, indicating a moderate external urethral sphincter activity before micturition (sphincter prevents voiding, as long as the storage reflex is active). During micturition, a high-amplitude low-frequency bursting, also called slow-wave-bursting, is most prominent (represents a peristaltic like propagation of the urine by the smooth muscles of the internal urethral sphincter). After micturition, high-amplitude high-frequency bursting is the most prominent pattern, showing a re-appearance of the storage reflex with strong contraction of the external urethral sphincter.

Figure 2

Metabolic cage tracing over 24 hours of weight of the voided urine, decreases by evaporation after each voiding and magnification of a single voiding



Urodynamic tracing of entire session and magnification of a single voiding: intravesical pressure, voided volume and external urethral sphincter EMG

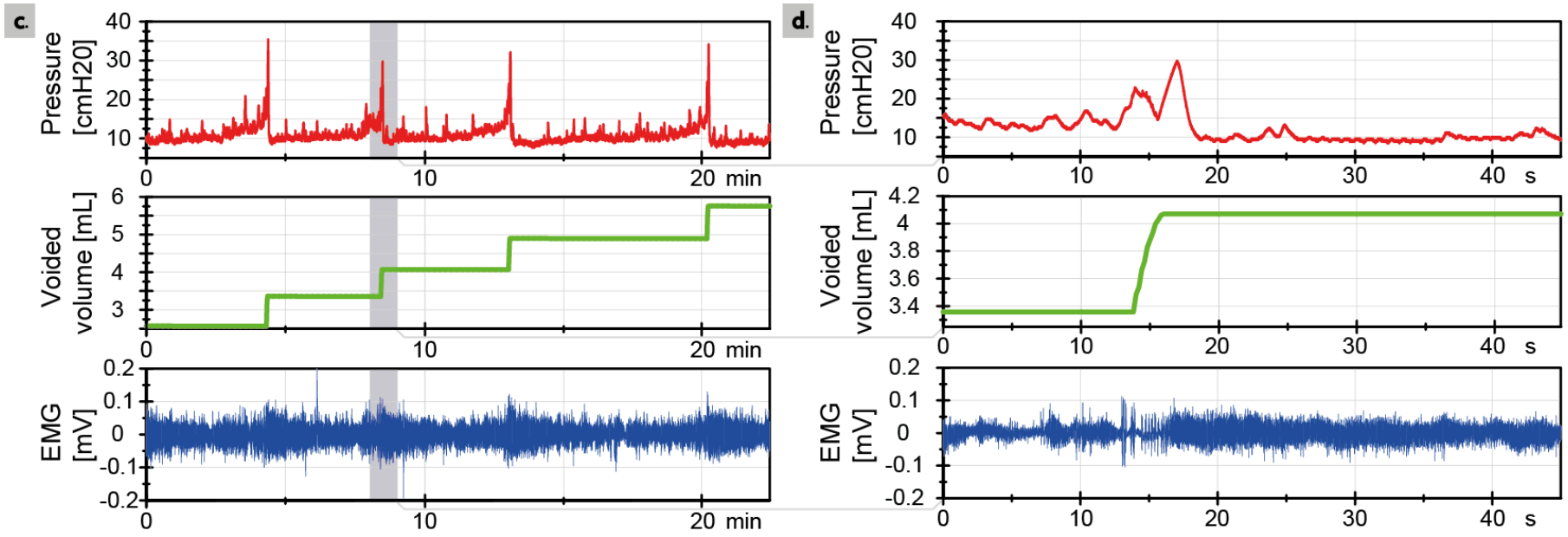


Figure 2: a. Metabolic cage tracing over 24 hours of weight of the voided urine, decreases by evaporation after each voiding (dark grey box represents “dark” phase, i.e. light off) and **b.** magnification (light gray box from a) of a single voiding. **c.** Urodynamic tracing of entire session and **d.** magnification (light gray box from c) of a single voiding: intravesical pressure, voided volume and external urethral sphincter EMG.

Quantitation of the urodynamic parameters voided volume, average flow and voiding duration are presented in Table 1 and Figure 3. Interestingly, they show no significant differences between the measurements in the metabolic cage and urodynamic measurements. No urinary tract infections, bladder stone formation, catheter- or electrode-related infections were observed. The animal weight remained constant (data not shown) during the entire course of the experiment.

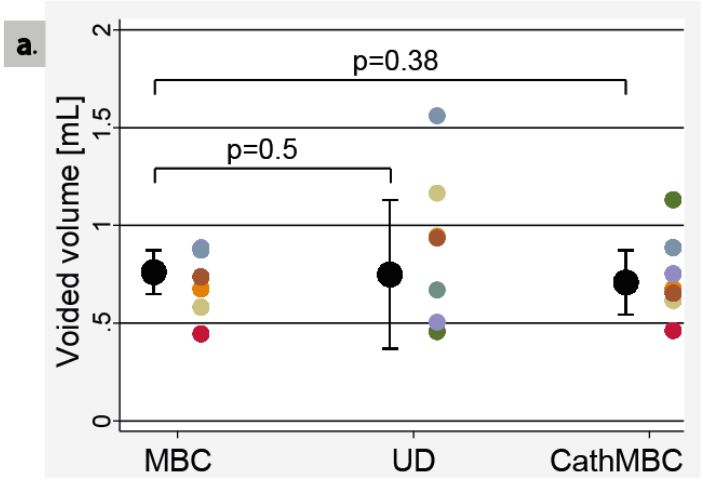
Table 1. Key parameters of micturition determined by urodynamic or metabolic cage

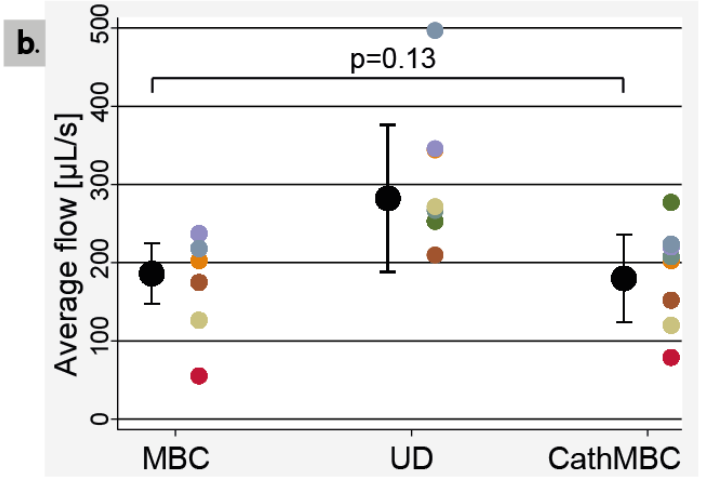
Condition	Light cycle	Number of animals	Mean number of micturitions per hour	Mean voided volume per void [mL]	Average flow during voiding [µL/s]	Average duration of voiding [s]
Urodynamics	light	8	10.6 (2.3)	0.748 (0.184)	282 (94)	2.6 (0.9)
Metabolic cage baseline in naïve animals	combined	27	0.8 (0.5)	0.747 (0.312)	193 (61)	3.5 (1.3)
	dark	27	1.1 (0.6)	0.715 (0.286)	170 (73)	3.5 (1.5)
	light	27	0.4 (0.4)	0.834 (0.353)	204 (72)	3.3 (1)
Metabolic cage 14 d after catheter implantation	combined	8	0.9 (0.3)	0.708 (0.164)	176 (75)	3.2 (0.9)
	dark	8	1.3 (0.5)	0.696 (0.196)	173 (82)	3.4 (1.1)
	light	8	0.7 (0.3)	0.725 (0.139)	179 (56)	3 (0.6)

All reported values represent means. Standard deviation is reported in parentheses. d = days; mL = milliliters; µL = microliters; s = seconds.

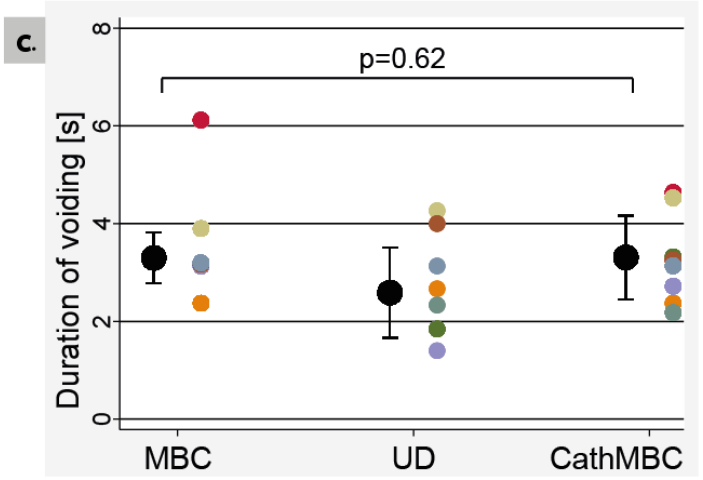
Figure 3

Comparison of three crucial parameters of micurition in three consecutive measurements: in naive rats in the metabolic cage (MBC), by urodynamics (UD), and in the catheter implanted rats by metabolic cage (cathMBC)





Comparison of voided volumes measured in metabolic cage during dark (active) and light (resting) diurnal phase



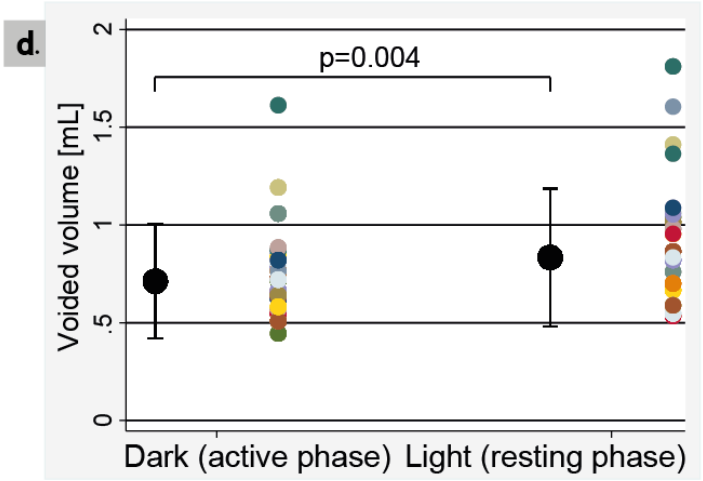


Figure 3: Comparison of three crucial parameters of micturition in the three consecutive measurements: in naive rats in the metabolic cage (MBC), by urodynamics (UD), and in the catheter implanted rats by metabolic cage (CathMBC): **a.** Voided volumes **b.** Average Flows **c.** Duration of voidings **d.** Comparison of voided volumes measured in metabolic cage during dark (active) and light (resting) diurnal phase. To avoid measurement bias, we only compared voiding duration and mean flow in metabolic cage naïve animals versus metabolic cage catheter implanted animals where the measurement situation is identical. For voided volumes, a correction formula was generated, allowing comparison of urodynamic and metabolic cage measurements.

4.5 Discussion

To the best of our knowledge, this is the first study comparing voiding parameters assessed by metabolic cage (naïve animals and catheter implanted animals) and by fully awake urodynamic model (catheter implanted animals). Our findings indicate that histological alterations in catheter implanted rats, observed in our previous study using exactly the same urodynamic model and timeline (Schneider, Hughes, et al., 2015), does not relevantly influence voided volume, average flow and duration of voiding. This implies that the urodynamic model in awake rats (Schneider, Hughes, et al., 2015) is currently the most appropriate translational approach to investigate lower urinary tract function. However, we observed significant differences between “light” and “dark” phase in voided volume assessed by metabolic cage.

Refractory lower urinary tract dysfunction remains a challenge in both neurological and non-neurological patients, since underlying patho-mechanisms are still not fully understood (Panicker et al., 2015; Schops et al., 2015). Thus, further basic research is highly warranted, in particular to identify new potential targets for first causal treatment approaches (Schneider, Schwab, et al., 2015). Truly translational animal models are therefore of great importance and the potential risk of systematic bias of specific models needs to be investigated in detail to increase the reproducibility and translational value of animal findings for human trials. Considering the convincing evidence (Schneider, Hughes, et al., 2015; Wuethrich et al., 2011; Wuethrich, Kessler, et al., 2013; Wuethrich, Metzger, et al., 2013) that bladder function is affected by anesthetics, animal models using anesthetics are problematic and the translational value highly questionable (Schneider, Hughes, et al., 2015). However, models with urodynamic measurements in awake rodents require a catheter implantation into the bladder and findings of previous studies (Andersson et al., 2011; Schneider, Hughes, et al., 2015) raised the question of a potential impact of catheter induced morphological changes in the bladder wall on normal lower urinary tract function. In the present study, we did not observe relevant differences. Thus, catheter implantation, although it may

lead to histological changes in the bladder wall, seems not to systematically alter bladder function in rats so that urodynamic measurements closely represent daily bladder function. Clinical data of indwelling suprapubic catheters (Kidd et al., 2015), a comparable situation for the bladder, support findings of our animal study underlying the truly translational approach of the urodynamic model in fully awake animals after chronic catheter implantation.

Comparing metabolic cage measurements during the “light” (i.e. animal resting state) versus the “dark” (i.e. animal active state) phase, we found significant differences in voided volumes. This raises the debate if urodynamic measurements should be performed in animals during the “dark” phase in order to be as translational as possible (Gammie et al., 2014). However, in rats (in contrast to humans), nocturia is physiological and almost as frequent as normal daytime voiding (Figure 2a), implying a comparable bladder function during “light” and “dark” phase. Nevertheless, further studies using an inverse cycle for animals are warranted, i.e. keeping animals and urodynamic equipment in darkened rooms with red-light (rats do not see red light, thus mimicking dark phase) and shifting the animals’ “light” phase from 6:00 a.m. until 6:00 p.m. to 6:00 p.m. until 6:00 a.m.

Main limitation of our study is that we investigated female rats only, to avoid confounding by prostatic bladder outlet obstruction causing voiding dysfunction particularly in older male animals. In addition, male rats do have an increased mortality rate after e.g. spinal cord injury due to prostatic bladder outlet obstruction causing urinary tract infections upon severe pyelonephritis with urosepsis. This is highly relevant in animal studies considering the 3Rs rule of Replacement, Reduction and Refinement (Flecknell, 2002; Kilkenny et al., 2010).

In conclusion, lower urinary tract function assessed by metabolic cage and urodynamic model in fully awake rats was similar. Thus, catheter implantation does not relevantly affect physiological bladder function, implying that the urodynamic model in awake animals is currently the most appropriate translational approach since it allows for

repetitive analysis at different time points in the same animal opening promising avenues to investigate lower urinary tract function. We will use this model in future studies, to investigate major neurological disorders causing lower urinary tract dysfunction like spinal cord injury (Liebscher et al., 2005), stroke (Wahl et al., 2014) and multiple sclerosis (Hiroya Mizusawa et al., 2000), where we expect it to provide better understanding of the patho-mechanisms involved.

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5 Anti-Nogo-A antibodies as a potential causal therapy for neurogenic lower urinary tract dysfunction after spinal cord injury

by **Marc P. Schneider**

Further contributions by:

Andrea Sartori, Benjamin V. Ineichen, Selina Moors, Anne K. Engmann, Oliver Weinmann, Martin E. Schwab and Thomas M. Kessler

M.P.S.: Concieved, initiated and designed the study (with help of A.K.E., T.M.K and M.E.S). Performed surgeries (with help of B.V.I, A.S., S.M.). Performed and analyzed urodynamics and metabolic cage (with help of A.S.). Established the digital workflows and extracted all data (with help of A.S.). Produced all figures (with help of A.S.). Wrote the manuscript (editing by A.S., B.V.I. and M.E.S).

In preparation for publication

5.1 Abstract

Detrusor sphincter dyssynergia is a common and severe complication of different neurological diseases including spinal cord injury. It is defined by dys-synergic contractions of the external urethral sphincter during voiding leading to high intravesical pressure, reflux of urine to the kidneys and finally life threatening kidney failure. Currently, no causal therapies are available to treat this dangerous condition.

Our objective was to investigate if antibodies against the nerve fiber growth inhibitory CNS protein Nogo-A applied to the injured spinal cord could prevent the development of lower urinary tract dysfunction, in particular detrusor sphincter dyssynergia, following spinal cord injury. Bladder function of female Lewis rats with complete or incomplete spinal cord injury at thoracic level 8 was repeatedly assessed using awake urodynamic examination enabled by implanted bladder catheters and external urethral sphincter electromyography electrodes.

Four weeks after large but incomplete thoracic spinal cord injury, detrusor sphincter dyssynergia had developed in all untreated or control antibody infused animals. In contrast, 2 weeks of intrathecal anti-Nogo-A-antibody treatment lead to a significantly reduced maximal detrusor pressure during voiding and a reduction of EMG high frequency activity in the external urethral sphincter. These data indicate that anti-Nogo-A-antibody treatment has beneficial effects on the lower urinary tract system, re-establising a physiological status and preventing detrusor sphincter dyssynergia after incomplete spinal cord injury, presumably by influencing the neuronal wiring of descending micturition circuits.

Anti-Nogo-A immunotherapy, which currently enters clinical trials for traumatic spinal cord injury, could, therefore, be a unique treatment optic also for lower urinary tract dysfunction, which is a severe, potentially life threatening condition in many diseases affecting the spinal cord.

5.2 Introduction

Many neurological diseases which affect descending tract systems, including spinal cord injury and multiple sclerosis, often lead to severe disturbances of bladder function. Detrusor sphincter dyssynergia (Weld et al., 2000), which is defined by dys-synergic contractions of the external urethral sphincter during the voiding phase, is a life-threatening complication as it leads to high intravesical pressure with subsequent urine reflux to the kidneys and, over years, renal failure (Groen et al., 2016). Impaired bladder control is among the strongest contributors for a decreased quality of life in the affected patients and therefore is one of the highest rehabilitation priorities (Simpson et al., 2012). Currently, there are only a few symptomatic treatment options available trying to treat this highly feared condition, like self-catheterization four to six times a day (Blok et al., 2016), Botox injections to weaken the respective muscles and anti-cholinergic medication. However, these treatments are at best symptomatic and come at the cost of frequent side effects such as urinary tract infections, or injuries to the urethra. Causal treatment options for neurogenic lower urinary tract dysfunctions, in particular detrusor sphincter dyssynergia, are therefore urgently needed (Panicker et al., 2015).

The CNS and myelin enriched membrane protein Nogo-A is a potent nerve fiber growth inhibitory protein, responsible in part for the low level of regeneration and repair present in the adult mammalian CNS(Schwab, 2010; Schwab & Strittmatter, 2014). Neutralization by antibodies or genetic deletion of Nogo-A induces substantial axonal regeneration, as well as enhanced neuronal plasticity and functional recovery after spinal cord injury or stroke in animal models (Liebscher et al., 2005; Wahl et al., 2014). Nogo-A can signal via various different receptors, the best described being NgR1 and S1PR2 (Kempf et al., 2014; Schwab & Strittmatter, 2014). Both of these receptors signal via the rho/ROCK pathway, ultimately causing growth cone collapse by destabilizing the actin cytoskeleton and downregulation of the neuronal growth program. It had been noted before that inhibition of Nogo-A in spinal cord injured rats

using function blocking antibodies not only resulted in improved recovery of locomotion, but also led to an earlier restoration of independent voiding (Liebscher et al., 2005). However, a detailed urodynamic assessment of bladder function after spinal cord injury to define the role of Nogo-A-antibodies as a potential therapy for detrusor sphincter dyssynergia following spinal cord injury has not been done so far.We recently established a urodynamic model which for the first time allows behavioral and electrophysiological assessment of bladder function in fully awake rats over several months (Schneider et al., 2015), thereby closely mimicking the urodynamic assessment in human patients in the clinical situation. The present study aims at investigating the effects of anti-Nogo-A-antibodies in the development of neurogenic lower urinary tract dysfunction, in particular detrusor sphincter dyssynergia, following a large spinal cord injury in adult rats

5.3 Materials and methods

Experimental design: The goal of the present study was to test the potential of anti-Nogo-A-antibodies to improve neurogenic lower urinary tract dysfunction, in particular detrusor sphincter dyssynergia after incomplete and complete spinal cord injury. To test this, the study had three parts: 1.) the quantification of dysfunction due to spinal cord injury in naïve rats, 2.) an explorative trial with anti-Nogo-A antibody to assess its effects on detrusor sphincter dyssynergia, and 3.) a comparative trial allowing after pre-hoc power calculation for unbiased statistical comparison of anti-Nogo-A antibody and control antibody treated rats.

All rats underwent the initial identical procedure with preoperative handling and acclimatization to the urodynamic lab station followed by catheter and electrode implantation. To minimize short lasting implantation-associated bladder dysfunction, healthy baseline urodynamics with simultaneous external urethral sphincter EMG measurements were not performed immediately but on postoperative day 10-14 (Andersson et al., 2011).

1.) All rats (n=32) for the quantitation of dysfunction due to spinal cord injury trial were randomly assigned to three different groups (no injury n= 17, incomplete spinal cord injury n= 8, complete spinal cord injury n= 7) after baseline urodynamic investigation. Rats were weekly assessed in the urodynamic lab station up to four weeks post spinal cord injury or equivalent time points in non-injured rats (Figure 1 a.).

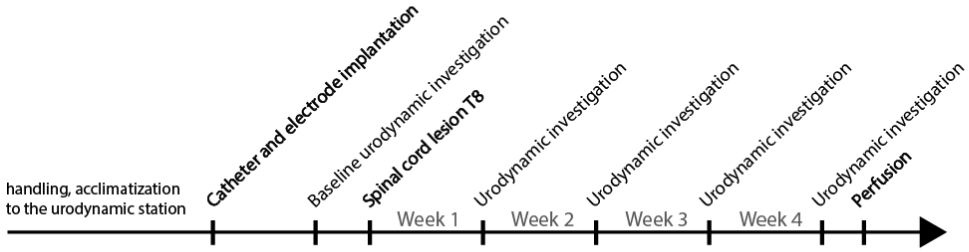
2.) All rats (n=34) of the explorative trial were treated according to the same protocol as the spinal cord injury groups except the additional implantation of a subcutaneous osmotic mini pump (model 2ML2, weight: 6 g, Alzet, USA) connected to a 32 gauge catheter inserted to the intrathecal space of the lumbar spinal cord. The pumps were either filled with an anti-Nogo-A-antibody (11C7, Novartis, Switzerland) or a control-antibody (anti-BrdU, Serotec). Following baseline urodynamic measurements, the rats were randomly divided in to four different groups (incomplete spinal cord injury with control antibody treatment n= 7, complete spinal cord injury with control antibody

treatment n= 10, incomplete spinal cord injury with anti-Nogo-A antibody treatment n= 6, complete spinal cord injury with anti-Nogo-A antibody treatment n= 11; Figure 1 b.). The lesion completeness was assessed *ex vivo* and group re-allocation was performed accordingly within the treatment groups.

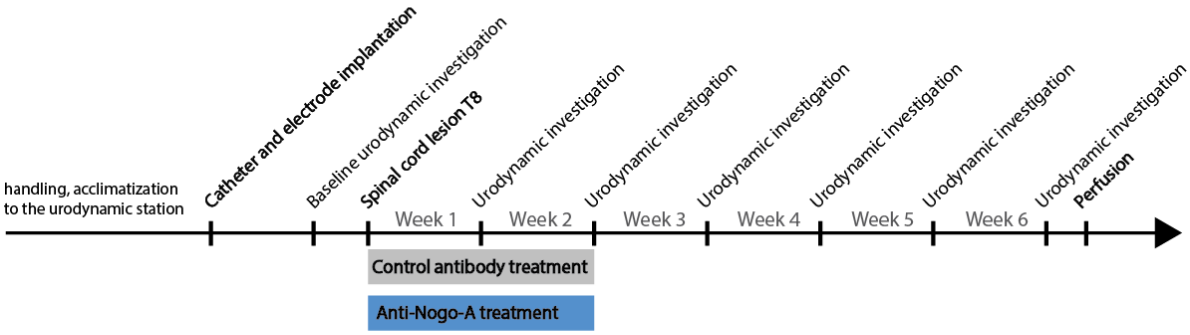
3.) A third comparative trial was planned and performed on the bases of observed effect sizes from trial two. Sample sizes were defined by pre-hoc power calculation (power = 80% and alpha = 0.05) and resulted in group sizes of n= 10 rats. Predefined primary outcomes (Figure 7 a.-l.) were only assessed at four weeks post spinal cord injury time point, to reduce the bias of multiple testing (Figure 1 c.).

Figure 1 - Experimental Timelines

a. Assessment of spinal cord injury impact on bladder function



b. Explorative trial



c. Comparative trial

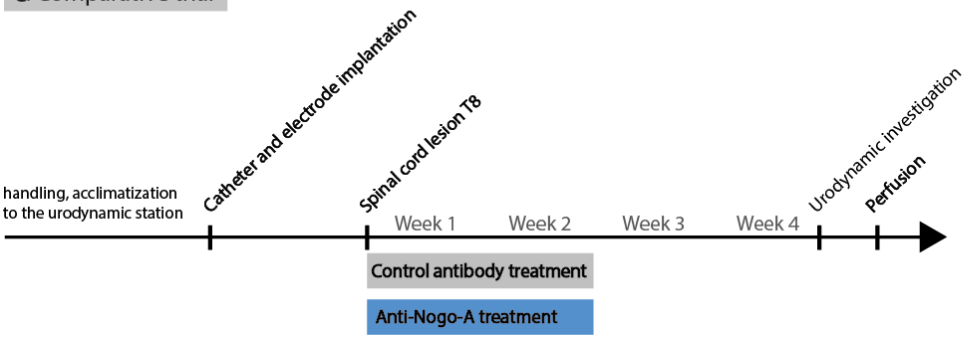


Figure 1 a. Study design of the trial to investigate the impact of spinal cord injury on bladder function (groups: naïve rats, incomplete spinal cord injury and complete spinal cord injury). **b.** Study design of the explorative trial on the treatment effect of anit-Nogo-A antibody therapy on neurogenic lower urinary tract dysfunction (groups: incomplete spinal cord injury treated with control antibodies, complete spinal cord injury treated with control antibodies, incomplete spinal cord injury treated with anti-Nogo-A antibodies and complete spinal cord injury treated with anti-Nogo-A antibodies). **c.** Study design of the comparative trial on the treatment effect of anti-Nogo-A antibody therapy on neurogenic lower urinary tract dysfunction (groups: incomplete spinal cord injury treated with control antibodies and incomplete spinal cord injury treated with anti-Nogo-A antibodies

Rats: Age-matched female Lewis rats (LEW/OrlRj (Lewis), 210 g ± 20 g, 4 mts ± 1mt, Janvier, France) were used in all studies. The rats were housed in groups of three to four per cage (single housed after catheter implantation), food and water were provided *ad libitum* (standard rat chow and water). Rats were maintained on a 12/12 h light/dark cycle (light on from 6:00 a.m. until 6:00 p.m). All experiments were approved by the Veterinary Office of the canton of Zürich, Switzerland (License nr. 19/2014) and were in accordance with the approved guidelines and regulations.

Catheter and electrodes implantation (as described previously (Schneider et al., 2015)): Briefly, rats were initially anesthetized in 5% Isoflurane (Piramal Healthcare) in air and maintained by an i.m. injection of Medetomidine (0.15 mg/kg Domitor, Orion Pharma), Midazolanum (2 mg/kg, Dormicum, Roche) and Fentanyl (5 µg/kg, Fentanyl, Kantonsapotheke University Hospital Zurich). Bladder catheters were inserted into the bladder dome and secured with a purse string suture. EMG electrodes were affixed to the fat tissue beside the external urethral sphincter and a ground electrode was sutured to the abdominal muscle. Catheter and wires were tunneled subcutaneously to the back of the neck, exteriorized, and lastly attached to an infusion harness (QC Single, SAI Infusion Technologies, USA).

Spinal cord injury (as described previously (Bachmann et al., 2013)): Rats were initially anesthetized in 5% Isoflurane (Piramal Healthcare) in air and maintained by an i.m. injection of Medetomidine (0.15 mg/kg Domitor, Orion Pharma), Midazolanum (2 mg/kg, Dormicum, Roche) and Fentanyl (5 µg/kg, Fentanyl, Kantonsapotheke University Hospital Zürich). T8 Lamina was removed. With a iridectomy scissors, the spinal cord was sectioned per experimental group (complete or severe but incomplete dorsal spinal cord injury).

Osmotic pump implantation (as described previously Ineichen et al., accepted): Briefly, in the same surgery as the spinal cord injury, laminectomy at vertebrate level L2 was performed and a fine intrathecal catheter (32 Gauge, ReCathCo) was inserted in to the subarachnoid space and pushed in cranial direction towards the lesion. The catheter

was connected to an osmotic mini pump (Alzet, model 2ML2, 5 µl/h, 3 mg/ml) which continuously delivered over 14 days either the anti-Nogo-A antibody (11C7 Novartis, Switzerland) or the control antibody (Anti-BrdU-antibody, Serotec) in to the intrathecal space. 6 mg antibodies were applied in total over 14 days. Pump and catheter were removed 15-16 days post implantation under 5% Isoflurane (Piramal Healthcare) anesthesia and the skin was closed by suture.

Urodynamic and external urethral sphincter EMG measurements (as described previously (Schneider et al., 2015)): Acclimatization of the rats to the urodynamic setup was performed before catheter and electrode implantation. Awake rats were positioned in a modified restrainer (modified from item # HLD-RM, Kent Scientific, Connecticut, USA) with a hole situated under the urethra. The restrainer was then placed in a modified Small Animal Cystometry Lab Station (Catamount Research and Development Inc.; St. Albans, Vermont, USA) with a scale below the funnel. The bladder catheter was attached to a syringe pump with an in-line pressure transducer and the electrodes were connected to an amplifier/converter. Saline was instilled (120 µL/min) and all parameters (pressure, scale, voltage) recorded simultaneously for at least 3 voiding cycles. Urodynamic measurements were performed during the light phase. For data assessment, the maximum voiding detrusor pressure was measured as the peak of the intravesical pressure during voiding (Figure 2.a). Voided volume was measured as the change in weight on the scale (assuming a density approaching 1). The threshold detrusor pressure was defined as the intravesical pressure just prior to the start of voiding. The lowest detrusor pressure, usually just after a voiding, was recorded as baseline detrusor pressure. To serve as a measurement of the rigidity of the bladder wall, bladder compliance was calculated as the intravesical pressure increase (threshold detrusor pressure minus baseline detrusor pressure) divided by the voided volume.

Blinding: Urodynamic and behavioral assessments were performed blinded by MPS and AS. BVI implanted the osmotic mini pumps and possessed the unbinding key, MPS and AS remained blinded until end of the experiment. Blinding of the urodynamic

readout was performed by a second person (MPS) who encrypted the animal's ID and the measuring time points. Urodynamic measurements were then analyzed blinded using a self-programmed LabVIEW program (AS). Unblinding was accomplished after data collection (MPS).

EMG analysis: The EUS EMG was bandpass filtered (2 Hz to 2 kHz). The frequency spectrogram was analyzed with a self-programmed application based on LabVIEW version 2012 (National Instruments, Austin, Texas, USA). The 45-second-long EMG signal is sliced into 4096 samples, of whom 3596 are overlapping (shift is 500 samples). After taking a hanning window of the signal, a fast Fourier transformation was generated. As result, we get a power value for each frequency at every computed time point. Before graphic processing, the signal was up scaled by a multiplication factor to assure that the signal consists, after logarithmizing, mainly of positive values. The logarithmized signal was also scaled up by factor 20. This signal was then plotted as color map with Jet Colormap. All frequencies above 500 Hz were neglected.

Perfusion and tissue preparation: Rats were euthanized with an i.p. overdose f of Pentobarbital (300mg/mL, Esconarkon ad us. Vet., Pentobarbitalumnatricum, Streuli Pharma AG). Followed by transcardial perfusion with 150mL Ringer solution (B.Brown Medical) containing 1% Heparin (B. Brown Medical) succeeded by 300 mL of 4% Paraformaldehyde solution (Paraformaldehyde, PFA, Sigma-Aldrich) containing 5% sucrose and phosphate-buffered at pH 7.4. Brain and spinal cord were dissected and after 18-20 hours of 4% Paraformaldehyde post fixation, transferred in to 30% sucrose and stored for three days for cryo-protection.

Assessment of lesion completeness (see also Supplementary Figure 1): Coronal 40-µm cryostat sections of the injury were manually reconstructed in a T8 spinal cord template based on a Nissl staining in Photoshop software (Adobe, San José, USA). Percentage of spared white matter was calculated after distraction of grey matter. Initial comparison of Nissl staining and neurofilament 160 immunohistochemical staining showed very high overlay in > 3% spared white matter sections (see also

Supplementary Figure 1). In rats with < 3% spared white matter we additionally stained to the Nissl staining for NF-160 allowing for more accurate estimation of spared white matter.

Statistical analysis: Data are reported as mean ± standard deviation (SD). Q-Q-plots were generated of all data and visually assessed, all data are assumed to be approximately normal distributed. For the comparison of the impact of spinal cord injury on bladder function, data was analyzed with a one-way repeated-measures ANOVA followed by Bonferroni's post hoc testing. For comparison of unrelated samples, an unpaired t-test was used in the comparative Nogo-A trial. The value of significance was considered at $p<0.05$. Pre-hoc power calculations were performed with alpha being 0.05 and a power of 80%. Statistical analyses were performed using STATA statistical software, version 14 (StataCorp, College Station, Texas, USA).

5.4 Results

Spinal cord injuries induce persistent deficits in urodynamic parameters (comparison of naïve, incompletely and completely injured animals): Acutely after the lesion all rats in the SCI groups described above showed almost complete initial paralysis of both hindlimbs as assessed by BBB (Basso et al., 1995). One week after injury, rats from the incomplete group developed spastic like movements of the hindlimbs. Incompletely lesioned animals recovered effective hind limb function (BBB`s score > 10) three weeks post spinal cord injury. Completely spinal cord injured animals remained with severe impairment of hind limb function (BBB`s score < 5) until the end of the experiment, i.e. up to six weeks post spinal cord injury. The impairments are comparable to human spinal cord injury patients with American Spinal Injury Association (ASIA) impairment scale (AIS) grade A for the complete spinal cord injury group and AIS grade C or D for the incomplete spinal cord injury group (www.ais.emsci.org).

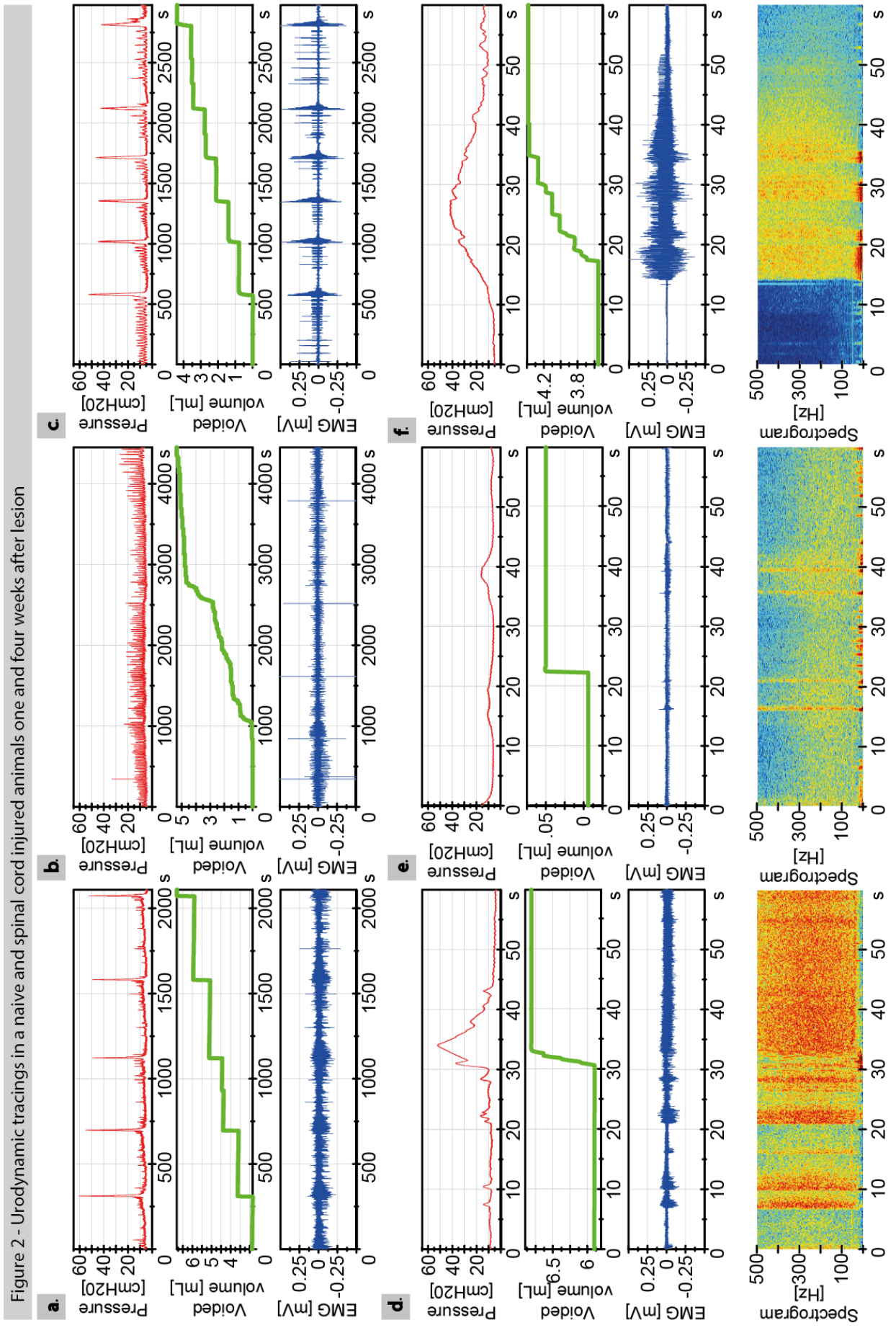


Figure 2 - Urodynamic tracings in a naive and spinal cord injured animals one and four weeks after lesion

Figure 2 DSD developes between 2 and 4 weeks after spinal cord injury. **a**. 40 min.(2200 sec.) and 1min. (60 sec.) windows of a representative urodynamic tracing from a naive rat with bladder catheter and external urethral sphincter EMG showing five voiding cycles at baseline. The top panel shows the bladder pressure tracing, the middle panel the secreted urine weight tracing and the bottom panel the external urethral sphincter EMG tracing. **b**. Spinal cord injured rat at one week after injury shows a flacid bladder. A 4500-s window of a representative urodynamic tracing from a rat with bladder catheter and external urethral sphincter EMG showing several voiding cycles at one week after spinal cord injury assessment, these voiding cycles are representative for any spinal cord injury rat in the spinal shock phase, regardless of group. The bladder contractions are insufficient to void, after an initial filling phase the bladder starts to leak, like dripping overflow incontinence. The top panel shows the bladder pressure tracing, the middle panel the secreted urine weight tracing and the bottom panel the external urethral sphincter EMG tracing. **c**.Typical characteristics of DSD at 4 weeks after injury. During the filling phase of the voiding cycle there are non-voiding contractions with increasing magnitude the fuller the bladder gets. These non-voiding contractions sometimes lead to incontinence i.e. this is an overactive bladder like situation. The top panel shows the bladder pressure tracing, the middle panel the secreted urine weight tracing and the bottom panel the external urethral sphincter EMG tracing. **d.** 60-s window culled from a. in a naïve animal. Markedly there is less external urethral sphincter EMG activity during voiding than before and after voiding. Top panel is the pressure tracing, middle panel the scale tracing and the bottom panel the external urethral sphincter EMG tracing. The heat plot at the bottom is a time matched frequency spectrogram where red represents a high power of the corresponding frequency at the current time point and blue represents low power. **e.** 60-s window culled from b. in a spinal cord injury animal at one week post lesion. Top panel is the pressure tracing, middle panel the scale tracing and the bottom panel the external urethral sphincter EMG tracing. The heat plot at the bottom is a time matched frequency spectrogram where red represents a high power of the corresponding

frequency at the current time point and blue represents low power. **f.** 60-s window culled from c. in a spinal cord injury animal at four weeks' post lesion. Markedly there is the highest external urethral sphincter EMG activity during voiding, in particular strong activity of high frequencies from 21-500 Hz are very different to normal situation and reflect a contraction of the striated muscle activity during voiding, i.e. detrusor sphincter dyssynergia. Top panel is the pressure tracing, middle panel the scale tracing and the bottom panel the external urethral sphincter EMG tracing. The heat plot at the bottom is a time matched frequency spectrogram where red represents a high power of the corresponding frequency at the current time point and blue represents low power.

Due to spinal shock, bladder function was completely absent during the first week and in contrast to locomotor performance subsequently only showed very limited recovery in all groups. Residual urine volume remained high in all spinal cord injured animals across the entire experiment (data not shown). All groups started with no difference among all urodynamic baseline parameters (Figure 3). Both spinal cord injury groups developed significant deficits ($p < 0.05$) in all pressure parameters after compared to before spinal cord injury (Figure 3 a.-f., maximal detrusor pressure during voiding, threshold detrusor pressure, average detrusor pressure, detrusor pressure during maximal flow, minimal detrusor pressure during voiding, maximal detrusor pressure). Interestingly, important pressure parameters such as average detrusor pressure, threshold detrusor pressure, minimal detrusor pressure during voiding and detrusor pressure at maximal flow continuously increased over time until four weeks post spinal cord injury, where they were above the level of the naïve control group. Both, the average flow rate and maximal flow rate were significantly reduced in both spinal cord injury groups but were even more decreased in the complete spinal cord injury group and did not recover over time (Figure 3 g./h.). Bladder compliance was significantly reduced at four weeks post spinal cord injury (Figure 3 i.). Voided volume acutely also was significantly reduced,showing a certain degree of recovery but remaining significantly below baseline values in both groups until the end of the experiment (Figure 3 j.). Voiding time, in contrast, was significantly increased over the complete experimental period even though a certain degree of recovery could be seen over time (Figure 3 k.). All these urodynamic findings are highly comparable to the human urodynamic situation after spinal cord injury and represent the human acute and early chronic stage after spinal cord injury (Panicker et al., 2015).

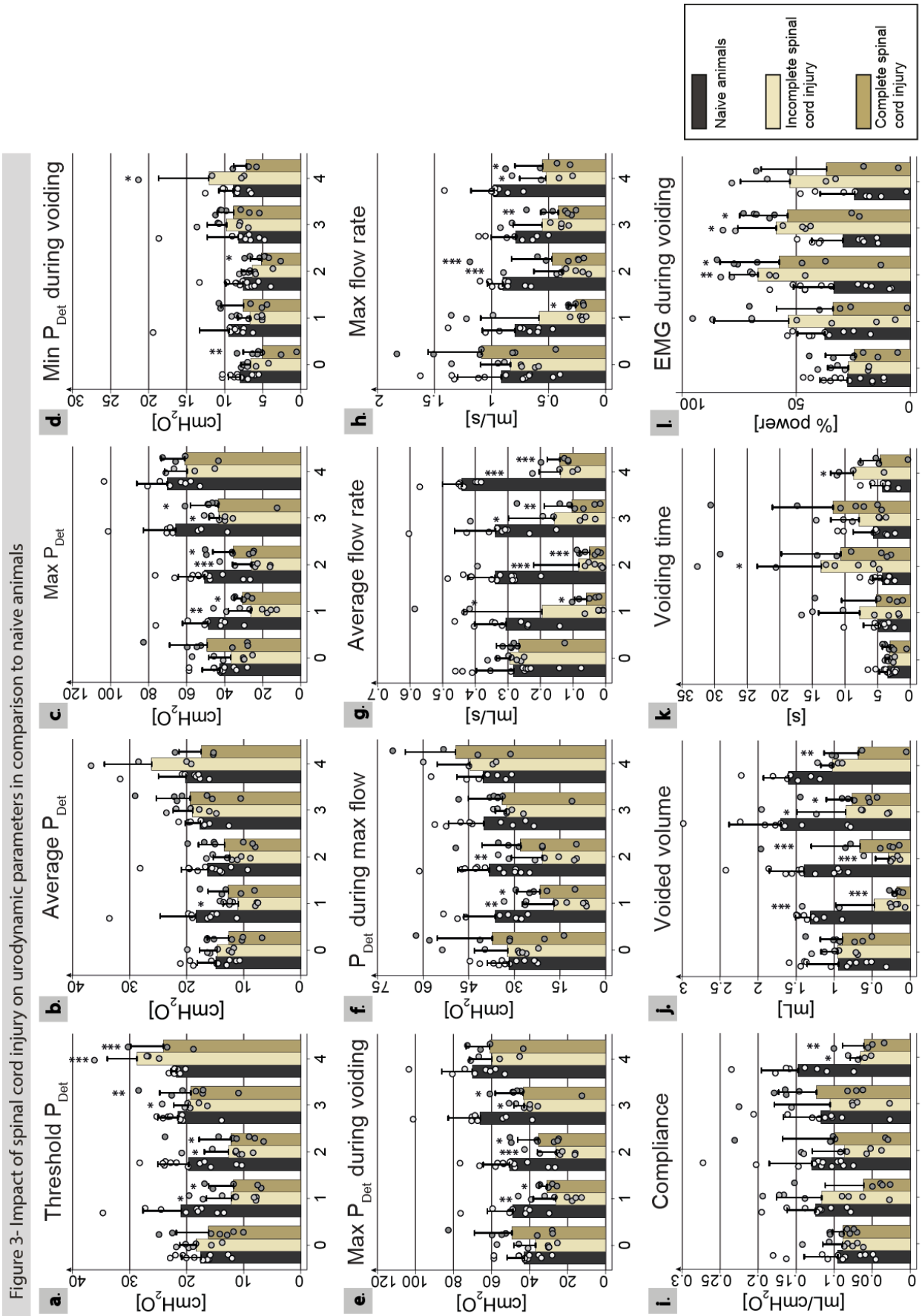


Figure 3- Impact of spinal cord injury on urodynamic parameters in comparison to naive animals

Figure 3 Urodynamic findings of trial one, assessing the impact of spinal cord injury on naive bladder function. **a**. Threshold detrusor pressure [cmH2O]; **b**. average detrusor pressure [cmH2O]; **c**. maximal detrusor pressure [cmH2O]; **d**. minimal detrusor pressure during voiding [cmH2O]; **e**. maximal detrusor pressure during voiding [cmH2O]; **f**. detrusor pressure during maximal flow [cmH2O]; **g**. average flow rate [mL/s]; **h**. maximal flow rate [mL/s]; **i**. bladder compliance [mL/cmH2O]; **j**. voided volume [mL]; **k**. voiding time [s]; **l**. EMG activity during voiding in comparison to before and after voiding [% total power]; Statistical testing: data of naïve and both complete and incomplete spinal cord injury was analyzed with a one-way repeated-measures ANOVA followed by Bonferroni's post hoc testing; *****= $p<0.05$; ******= $p<0.01$ and *******= $p<0.001$.

Spinal cord injuries induce persistent changes in external urethral sphincter EMG: In naive rats, the external urethral sphincter showed strong peak activity in the high frequency range (21-500Hz, representing striated muscle activity) at the high filling state immediately before voiding, as well as after voiding. High frequency activity was low during voiding (Figure 2 a. and Figure 4 a.), where in contrast, slow wave bursting activity (2-20Hz, representing smooth muscle activity) was prominent (Figure 2 a. and Figure 4 a.).

16 out of 17 rats with either complete or incomplete spinal cord injury developed detrusor sphincter dyssynergia, starting 2-3 weeks post spinal cord injury, characterized by high tonic high frequency sphincter EMG activity (21-500Hz, representing striated muscle activity) during the voiding phase, which resulted in dripping and interrupted urine release and highly prolonged voiding phases (Figure 2 c., Figure 3 j. and Figure 4 b.). These characteristics again closely resemble the situation seen in severely spinal cord injured human patients.

In naïve rats, high frequency (21-500Hz) EMG activity during voiding was most prominent in the initial quarter of voiding. The following three quarters showed only a fraction of the initial peak EMG activity, representing the initial presence of the guarding reflex which is subsequently efficiently inhibited leading to a relaxation of the external urethral sphincter, hence allowing efficient voiding (Figure 4 c.). In contrast, both the complete and incomplete spinal cord injury animals displayed 3-4-fold increased EMG power amplitudes during the second quarter of the voiding period, showing a reduced inhibition of the guarding reflex (Figure 4 d./e.).

In naïve animals, low frequency (2-20Hz) EMG activity could be observed over the whole course of the voiding period (Figure 4 f.). Contrastingly, in both complete and incomplete spinal cord injury groups low frequency EMG power was reduced towards the last quarter of the voiding period (Figure 4 g./h.).

Most interestingly, the percental high frequency average power before, during and after voiding in naïve animals was at about equal levels with a trend of the strongest power

before voiding (Figure 4 i.). This was completely changed in the injured situation (complete and incomplete) where the percental high frequency average power was most prominent during voiding (Figure 4 j./k.), i.e. this stands for increased external urethral sphincter activity during voiding and is diagnostic for detrusor sphincter dyssynergia.

Urodynamic and external urethral sphincter parameters are not influenced by lesion completeness: No significant differences were found in any urodynamic parameter when incomplete and complete spinal cord injured animals were compared (Figure 3 a.-l.). Also, we did not find any differences comparing both high and low frequency activity of the external urethral sphincter EMG during voiding (Figure 4 d./e./g./h.) of incomplete and complete spinal cord injury groups. In contrast, we observed a trend towards a less severe activity of the external urethral sphincter during voiding in the complete spinal cord injury animals (Figure 4 j./k.).

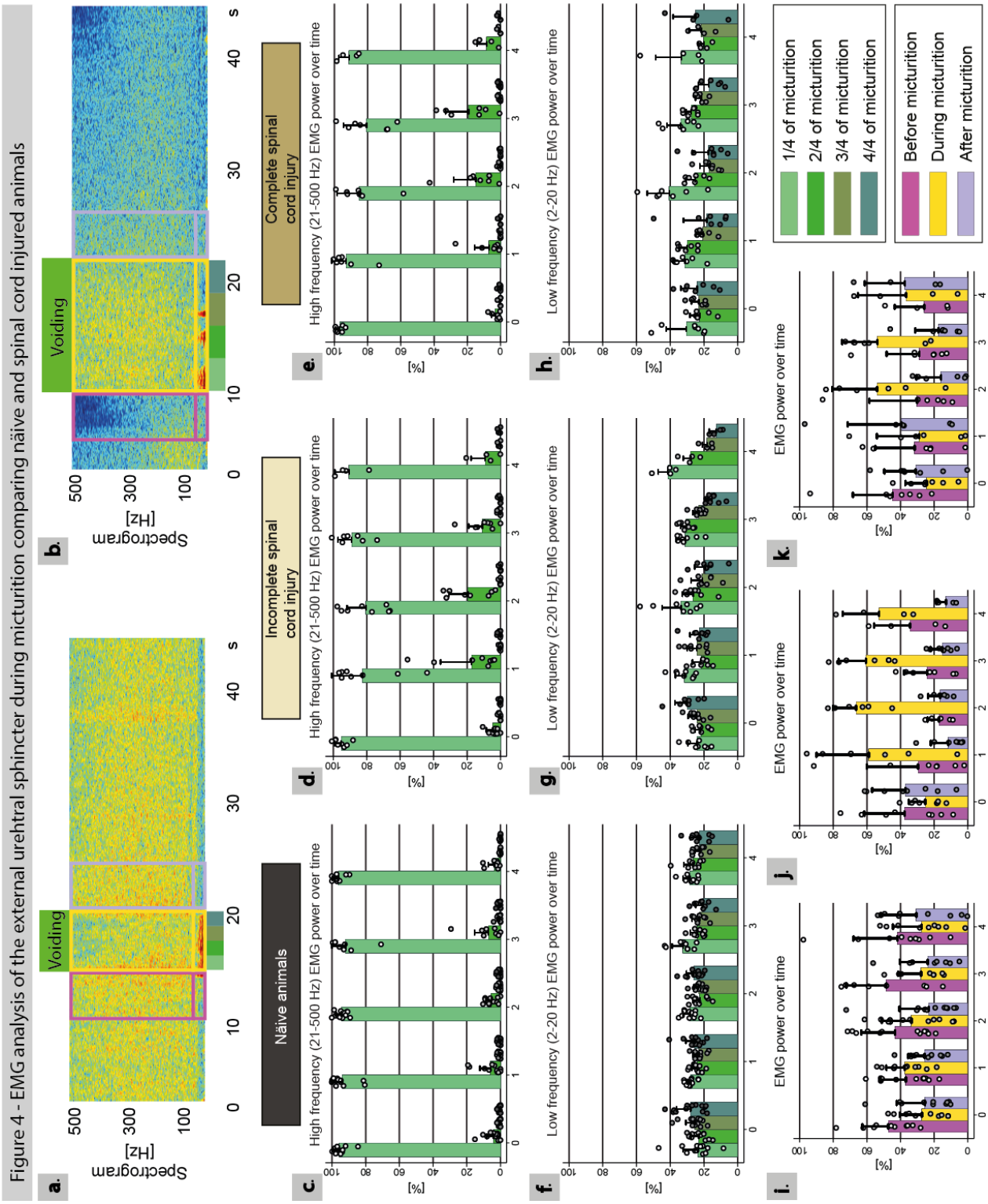


Figure 4 - EMG analysis of the external urethral sphincter during micturition comparing näive and spinal cord injured animals
a.
b.
Voiding
Spectrogram [Hz]
500
300
100
0
10
20
30
40
s
Näive animals
Incomplete spinal cord injury
Complete spinal cord injury
c.
d.
e.
f.
g.
h.
High frequency (21-500 Hz) EMG power over time
Low frequency (2-20 Hz) EMG power over time
[%]
i.
j.
k.
EMG power over time
1/4 of micturition
2/4 of micturition
3/4 of micturition
4/4 of micturition
Before micturition
During micturition
After micturition

Figure 4 External urethral sphincter EMG analysis of trial one, assessing the impact of spinal cord injury on naive bladder function. **a**. Spectrogram of a voiding of a naïve rat; **b**. Spectrogram of a voiding of an incomplete spinal cord injury rat four weeks after spinal cord injury; **c**. High frequency power (21-500Hz) during voiding plotted for four quarters of the voiding in naïve rats. **d**. High frequency power (21-500Hz) during voiding plotted for four quarters of the voiding of incomplete spinal cord injury rats. **e**. High frequency power (21-500Hz) during voiding plotted for four quarters of the voiding of incomplete spinal cord injury rats. **f**. Low frequency power (2-20Hz) during voiding plotted for four quarters of the voiding in naïve rats. **g**. Low frequency power (2-20Hz) during voiding plotted for four quarters of the voiding of the voiding of incomplete spinal cord injury rats. **h**. Low frequency power (2-20Hz) during voiding plotted for four quarters of the voiding of the voiding of complete spinal cord injury rats. **i**. Average high frequency power activity (21-500Hz) of the external urethral sphincter comparing before during and after voiding in naive rats. **j.** Average high frequency power activity (21-500Hz) of the external urethral sphincter comparing before during and after voiding of incomplete spinal cord injury rats. **j.** Average high frequency power activity (21-500Hz) of the external urethral sphincter comparing before during and after voiding of complete spinal cord injury rats.

In an explorative trial, anti-Nogo-A antibody treated animals showed a persistent trend towards a treatment effect in incomplete spinal cord injury animals compared to incomplete control antibody treated animals. No effect was observed in complete spinal cord injury groups: No statistical testing was made in this explorative pilot trial between the groups. Comparing anti-Nogo-A antibody treated with control antibody treated animals, several parameters were permanently reduced starting three weeks' post spinal cord injury (Figure 5 a.-k.). Percental reduction in the anti-Nogo-A antibody treated group at four weeks' post spinal cord injury time point when compared to the pre-injury baseline values: voided volume (34%), voiding time (62%), maximal detrusor pressure during voiding (51%), threshold detrusor pressure (46%), average detrusor pressure (41%), detrusor pressure during maximal flow (55%), minimal detrusor pressure during voiding (37%), maximal detrusor pressure (50%), compliance (46%), maximal flow rate (32%). The percental high frequency average power during voiding showed the biggest change, it was decreased by 73% in the injured setting (Figure 5 l.).

Post hoc power calculation was performed based on observed anti-Nogo-A antibody treatment effect sizes and corresponding variabilities for a power of 80% and alpha being 0.05 at the four weeks' post spinal cord injury time point for important parameters and resulted in the following estimated required sample sizes: Maximal detrusor pressure n= 5, voided volume n= 7 and voiding time n= 4. The group size for the subsequent comparative trial was therefore set to n= 10.

No treatment effects could be observed in completely spinal cord injured animals treated with control and anti-Nogo-A antibody (Figure 5 a.-l.).

Figure 5 - Explorative study on treatment effect of anti-Nogo-A antibody in neurogenic lower urinary tract dysfunction due to spinal cord injury

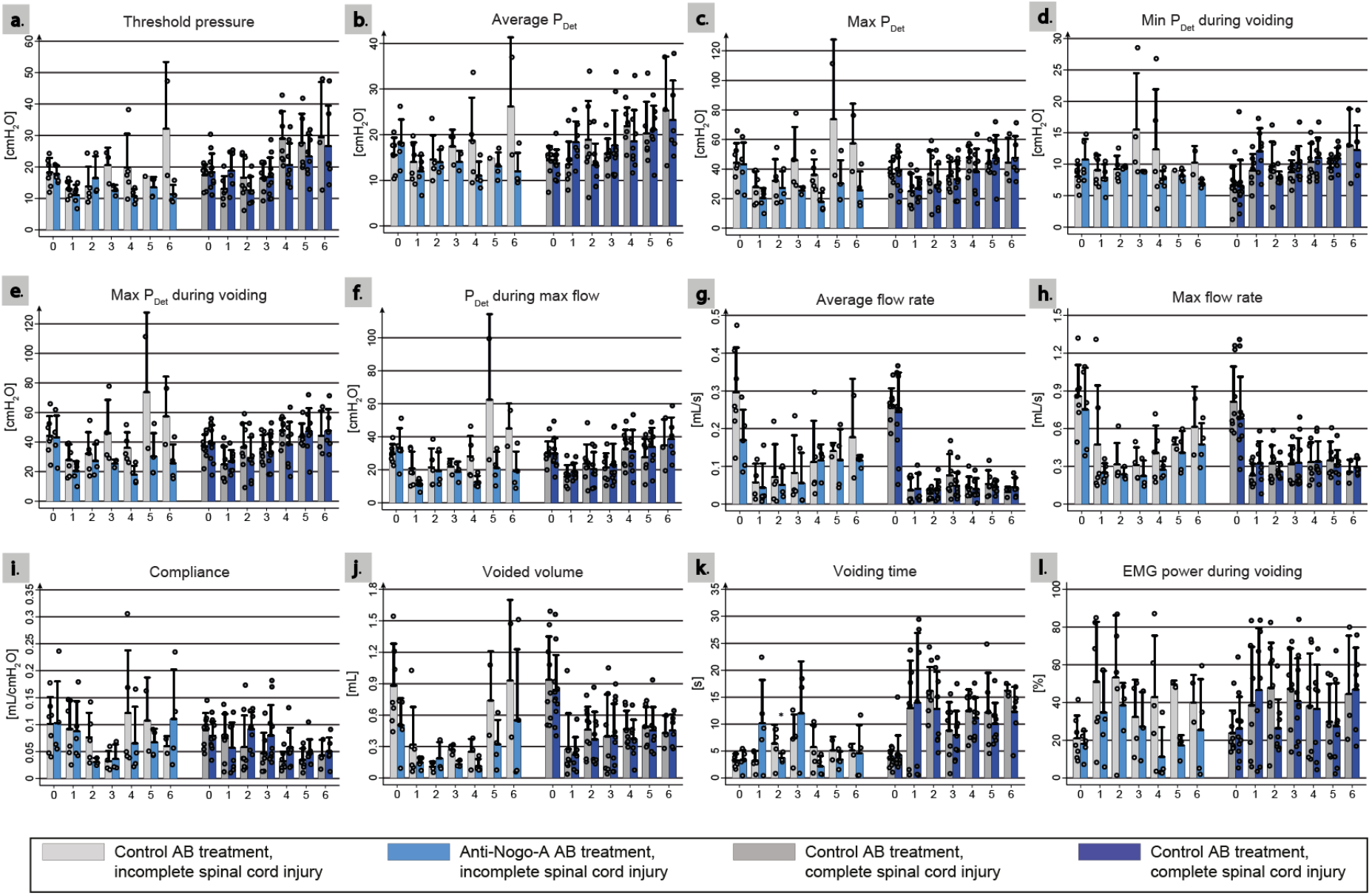


Figure 5 Urodynamic findings of trial two, exploring the potential treatment effect of anti-Nogo-A antibody on neurogenic lower urinary tract dysfunction, in particular detrusor sphincter dyssynergia following spinal cord injury. **a**. Threshold detrusor pressure [cmH2O]; **b**. average detrusor pressure [cmH2O]; **c**. maximal detrusor pressure [cmH2O]; **d**. minimal detrusor pressure during voiding [cmH2O]; **e**. maximal detrusor pressure during voiding [cmH2O]; **f**. detrusor pressure during maximal flow [cmH2O]; **g**. average flow rate [mL/s]; **h**. maximal flow rate [mL/s]; **i**. bladder compliance [mL/cmH2O]; **j**. voided volume [mL]; **k**. voiding time [s]; **l**. EMG activity during voiding in comparison to before and after voiding [% total power]; No statistical testing since it was an explorative trial.

Anti-Nogo-A antibody treatment improved external urethral sphincter function during voiding and prevented development of detrusor sphincter dyssynergia in incomplete spinal cord injured rats: In the anti-NogoA and Crl antibody treated incomplete spinal cord injury groups, high frequency (21-500Hz) EMG activity during voiding showed very prominent activation at the initial quarter of voiding. The following three quarters showed only a few percent of the initial peak activity (Figure 6 a./b.). In contrast, both (anti-Nogo and crl antibody) complete spinal cord injury groups have EMG power amplitude increases by 3-4 folds during the 2/4 of voiding, less prominent in the complete anti-Nogo-A antibody treated group. Indicating a reduced inhibition of the guarding reflex, i.e. a contraction of the external urethral sphincter muscle during voiding (Figure 6 c./d.).

In anti-Nogo-A antibody treated incomplete spinal cord injury rats, low frequency (3-20Hz) EMG activity during voiding showed very equal activation over the entire voiding (Figure 6 f.) similar to healthy naïve situation. In particular, slow wave bursting is still prominent at the end of voiding. Contradictory, in control antibody treated incomplete spinal cord injury rats is the low frequency EMG power reduced towards the last quarter of voiding (Figure 6 e.), similar to the not treated incomplete spinal cord injury rats.

Most interestingly, in anti-Nogo-A antibody treated incomplete spinal cord injury rats is the percental high frequency average power before, during and after voiding at equal levels with a trend of the strongest power before voiding (Figure 6 j.). This precisely reflects the normal healthy and naïve situation with now detrusor sphincter dyssynergia, whereas the control antibody treated animals developed same severe detorsor sphincter dyssynergia, reflected by stronger percental high frequency average power during voiding than before and after. This shows that anti-Nogo-A antibody therapy may prevent detrusor sphincter dyssynergia at all. A comparative trial was planned to prove the findings from this explorative trial.

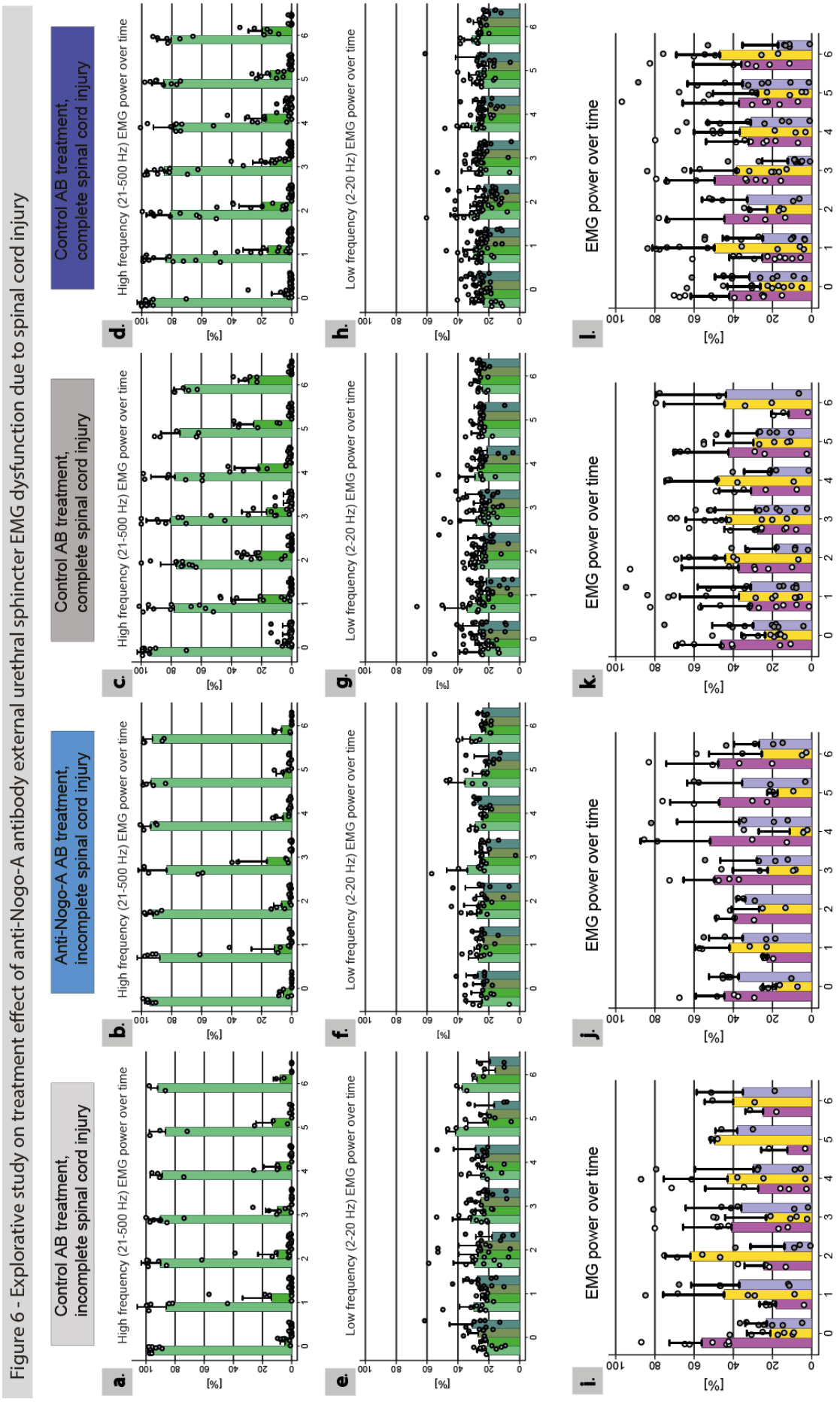


Figure 6 - Explorative study on treatment effect of anti-Nogo-A antibody external urethral sphincter EMG dysfunction due to spinal cord injury

Figure 6 External urethral sphincter EMG analysis of trial two, exploring the treatment effect of anti-Nogo-A antibody therapy for neurogenic lower urinary tract dysfunction of spinal cord injury on naive bladder function. **a**. High frequency power (21-500Hz) during voiding plotted for four quarters of the voiding in control antibody treated animals with incomplete spinal cord injury rats, **b**. control antibody therapy with complete spinal cord injury rats. **c**. anti-Nogo-A antibody therapy with incomplete spinal cord injury rats. **d**. anti-Nogo-A antibody therapy with complete spinal cord injury rats. **e**. Low frequency power (2-20Hz) during voiding plotted for four quarters of the voiding in control antibody treated animals with incomplete spinal cord injury rats, **f**. control antibody therapy with complete spinal cord injury rats, **g**. anti-Nogo-A antibody therapy with incomplete spinal cord injury rats, **h**. anti-Nogo-A antibody therapy with complete spinal cord injury rats. **i.** Average high frequency power activity (21-500Hz) of the external urethral sphincter comparing before during and after voiding in control antibody treated animals with incomplete spinal cord injury rats, **j**. control antibody therapy with complete spinal cord injury rats, **k**. anti-Nogo-A antibody therapy with incomplete spinal cord injury rats, **l**. anti-Nogo-A antibody therapy with complete spinal cord injury rats.

Anti-Nogo-A antibody therapy significantly reduced important urodynamic parameters in incomplete spinal cord injury animals when compared to incomplete control antibody treated animals: Four weeks after spinal cord injury we found significant effects in the following urodynamic parameters when incompletely lesioned animals treated with the anti-Nogo-A antibody were compared to respective lesioned control animals (Figure 7 a.-l.): voided volume ($p= 0.002$), maximal detrusor pressure during voiding ($p= 0.01$), average flow rate ($p= 0.006$), average detrusor pressure ($p= 0.01$), detrusor pressure during maximal flow ($p= 0.01$), maximal detrusor pressure ($p= 0.01$), compliance ($p= 0.006$), maximal flow rate ($p= 0.05$). Most impressively was again the reduction of the percental high frequency average power during voiding ($p= 0.002$). All changes were in the same direction and of comparable magnitude as the explorative initial trial.

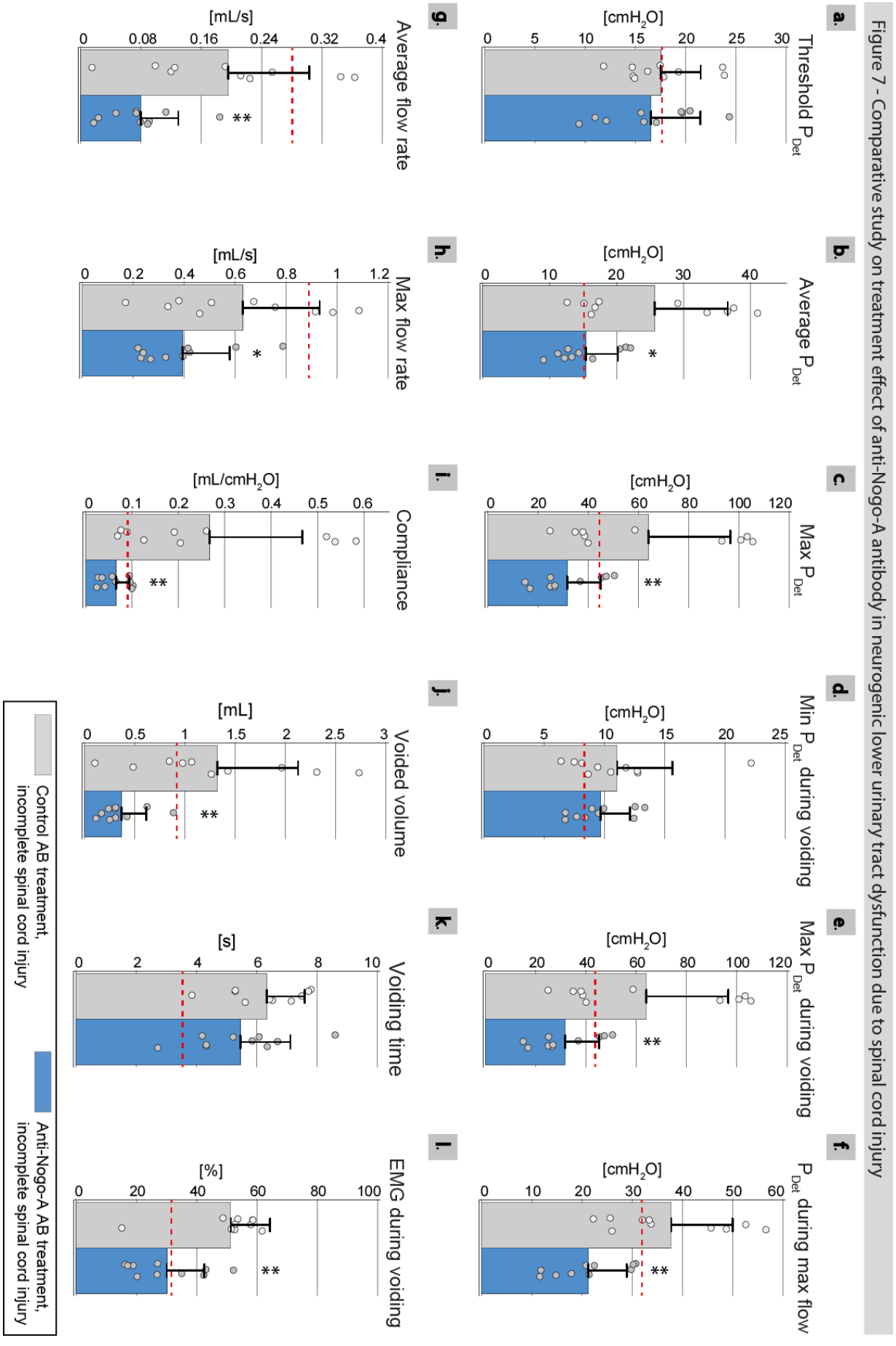


Figure 7 - Comparative study on treatment effect of anti-Nogo-A antibody in neurogenic lower urinary tract dysfunction due to spinal cord injury

Figure 7 Urodynamic findings of trial three, assessment of the treatment effect of anti-Nogo-A antibody on neurogenic lower urinary tract dysfunction, in particular detrusor sphincter dyssynergia following incomplete spinal cord injury. **a**. Threshold detrusor pressure [cmH2O]; **b**. average detrusor pressure [cmH2O]; **c**. maximal detrusor pressure [cmH2O]; **d**. minimal detrusor pressure during voiding [cmH2O]; **e**. maximal detrusor pressure during voiding [cmH2O]; **f**. detrusor pressure during maximal flow [cmH2O]; **g**. average flow rate [mL/s]; **h**. maximal flow rate [mL/s]; **i**. bladder compliance [mL/cmH2O]; **j**. voided volume [mL]; **k**. voiding time [s]; **l**. EMG activity during voiding in comparison to before and after voiding [% total power]; Statistical testing: data of anti-Nogo-A antibody treated rats and control antibody treated rats was analyzed with an unpaired students t-test; *****= $p<0.05$; ******= $p<0.01$ and *******= $p<0.001$. Red dashed line reflects the values of the healthy control group.

Anti-Nogo-A antibody significantly reduced external urethral sphincter activity during voiding in incomplete spinal cord injury animals compared to incomplete control antibody treated animals: Four weeks after spinal cord injury we found significant effects in the percental high frequency average power during voiding ($p= 0.002$) comparing anti-Nogo-A antibody treated SCI rats and control antibody treated animals (Figure 8 c.). In anti-Nogo-A antibody treated incompletely injured rats, low frequency (2-20Hz) EMG activity during voiding showed very equal activation over the entire voiding (Figure 8 b.) like the healthy naïve situation (Figure 4 f.). In particular, upon anti-NogoA therapy, slow wave bursting of the external urethral sphincter was still present at the end of voiding. Contrastingly, in control antibody treated incomplete spinal cord injury rats is the low frequency EMG power declined towards the last quarter of voiding (Figure 8 b.), like in not treated incomplete spinal cord injury rats (Figure 4 g.) All changes were in the same direction and of comparable magnitude as the explorative initial trial.

Figure 8 - Treatment effect of anti-Nogo-A antibody on external urethral sphincter EMG activity

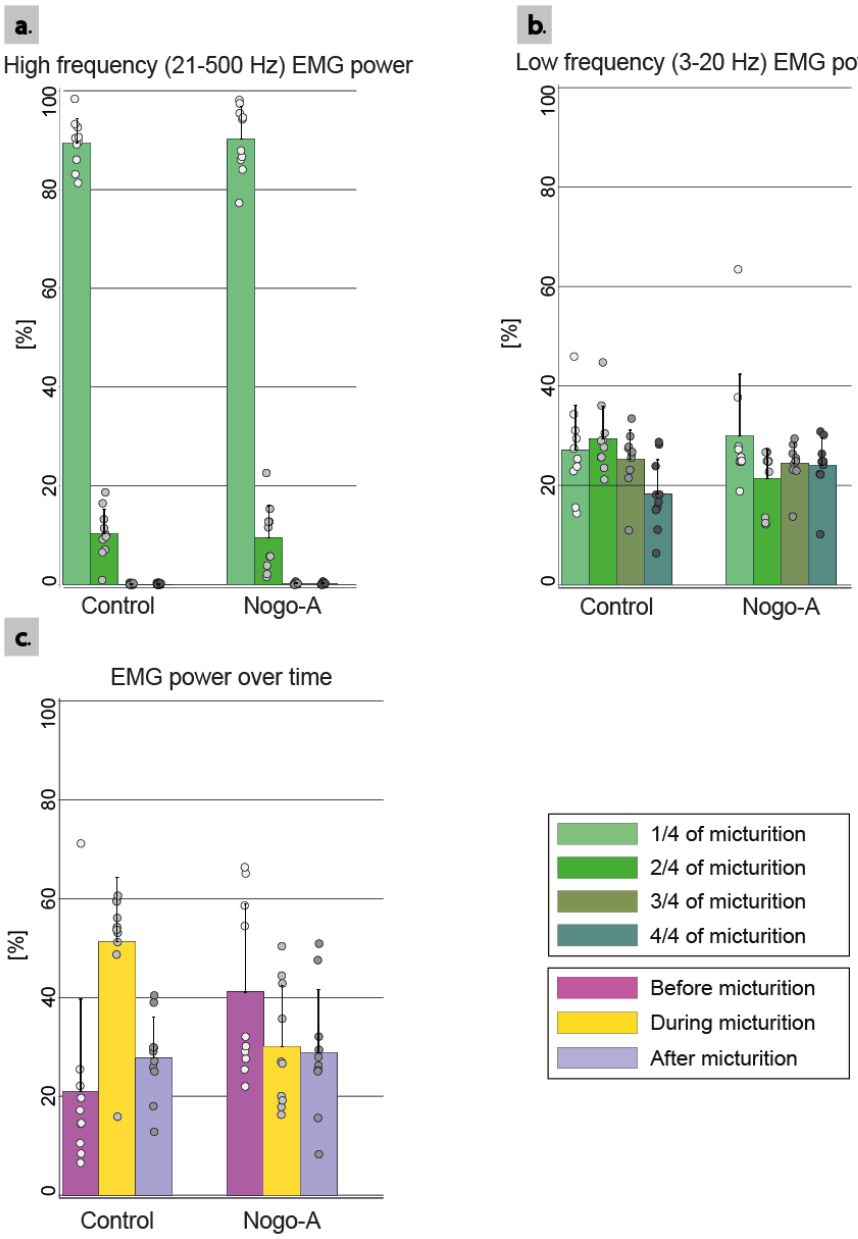


Figure 8 External urethral sphincter EMG analysis of comparative trial three, comparing the treatment effect of anti-Nogo-A antibody therapy with control antibody therapy for neurogenic lower urinary tract dysfunction of spinal cord injury on naive bladder function at four weeks after spinal cord injury. **a**. High frequency power (21-500Hz) during voiding plotted for four quarters of the voiding. **b**. Low frequency power (2-20Hz) during voiding plotted for four quarters of the voiding **c**. Average high frequency power activity (21-500Hz) of the external urethral sphincter compared before during and after voiding.

5.5 Discussion

To the best of our knowledge, this is the first study investigating the treatment effect of anti-Nogo-A antibody on neurogenic lower urinary tract dysfunction, in particular detrusor sphincter dyssynergia caused by spinal cord injury. Our data show strong similarities of pathological changes seen in the urodynamic assessment in rats compared to humans with spinal cord injury (Schops et al., 2015; Weld et al., 2000). Most prominent is the massive increase in EMG activity of the external urethral sphincter. This is the hallmark for the life threatening detrusor sphincter dyssynergia, defined by dys-synergistic contraction of the external urethral sphincter during voiding leading to increased bladder pressure and eventually urinary reflux to the kidneys (Panicker et al., 2015). Our findings in rats are highly comparable to spinal cord injured humans commonly affected by detrusor sphincter dyssynergia situation after the lesion (Fowler et al., 2008). An explorative trial with intrathecal anti-Nogo-A antibody treatment after incomplete spinal cord injury showed normalization in several urodynamic parameters at 4 weeks post lesion, including the EMG activity of the external urethral sphincter during voiding which recovered nearly back to baseline values, in comparison with control antibody treated animals (Figures 5 and 6). No treatment effect was observed in completely spinal cord injured animals treated with anti-Nogo-A antibody. A comparative trial, planned upon stringent power calculations based on the explorative trial showed similar results with strong significant normalization of several urodynamic parameters including the EMG activity of the external urethral sphincter during voiding, four weeks after spinal cord injury.

In human spinal cord injury patients, the bladder function is completely out of control and detrusor sphincter dyssynergia is a common and greatly feared complication of this pathological condition (Fowler et al., 2008). The development of neurogenic lower urinary tract dysfunction after spinal cord injury follows a typical time line defined by an initial spinal shock phase with the detrusor and sphincter muscle being both paralyzed resulting in high residual urine volumes, followed by detrusor over activity after some

months (Panicker et al., 2015). In most cases, this leads to urinary incontinence. Detrusor sphincter dyssynergia, the most dangerous dysfunction pattern, develops concurrently with the detrusor over activity (Abrams, 2003). It is defined by simultaneous contractions of the external urethral sphincter during detrusor contraction for voiding (Abrams et al., 2002). This leads to a steady increase of intravesical pressure over years due to compensatory hypertrophy of the detrusor muscle (Blok et al., 2016). This increased pressure system leads to renal reflux and, in combination with urinary tract infections, potentially culminates in complete loss of kidney function (Groen et al., 2016). We observed a similar course of events after complete or incomplete thoracic spinal cord injury in rats with an approximately four folds' faster time course in our trial, i.e. one week in rats corresponds approximately to one month in humans (Kaplan et al., 2015). The rats developed similar detrusor sphincter dyssynergia (Figure 2c./f.) like humans after the initial spinal shock phase (Figure 2 b./e.) of approximately one to two weeks. Due to methodological (clogging of catheters, increasing urinary tract infections and broken electrodes) and ethical limitations (overall burden for the rats) of our experimental setup, we followed up to six weeks post spinal cord injury corresponding to the early chronic phase in humans. Therefore, increased intravesical pressures start to develop at our later investigated time points in the rats. The earliest time point for which we observe this increase in bladder pressure is four weeks after spinal cord injury, as shown in Figure 3 b.-d.; values of threshold-, average- and detrusor pressure during maximal flow are outstripping the values from the naïve animals, hinting towards increased bladder pressure systems for more chronic time points as it is the case for humans. Conclusively, our urodynamic model in fully awake rats can demonstrate the pathological impact of spinal cord injury on the lower urinary tract. The observed changes are very like the situation in humans emphasizing the translational value of this approach.

Liebscher and colleagues found that thoracic level 8 incomplete spinal cord injury animals treated with anti-Nogo-A antibody in a very similar study setting like ours, could void their bladders independently 15 days post spinal cord injury compared to control

antibody treated animals which needed up to 25 days to restore this function (Liebscher et al., 2005). Based on these observations, the current study was initiated and we found that anti-Nogo-A antibody therapy had a strong effect on bladder function recovery after thoracic spinal cord injury defined by several important urodynamic pressure values. But most importantly, the anti-Nogo-A antibody treatment led to a restoration of the physiological external urethral sphincter function during voiding. Antibodies against Nogo-A were infused in our trial intrathecally of the lumbar spinal cord. These antibodies distribute along the complete cerebrospinal axis via circulation in the cerebrospinal fluid (Weinmann et al., 2006). After binding of this antibody to Nogo-A, which is mainly expressed on oligodendrocytes in the adult central nervous system, the antibody-Nogo-A-complex is being internalized with subsequent downregulation of surface Nogo-A (Weinmann et al., 2006). Downregulation of this surface Nogo-A in combination with the blocking of the active side by the anti-Nogo-A antibody is probably an important aspect of the mechanism of action (Liebscher et al., 2005). These sequence of processes leads to a decreased Nogo-A-signaling to neurons mediated by NgR1. Thereby, the CNS is shifted towards a more plastic environment enabling more efficient axonal regeneration after injury. Proven by the substantial functional improvements, it can be concluded that these additional fibers and synapses bind to meaningful targets. Anti-Nogo-A antibody treatment has been shown to induce regeneration and sprouting in the corticospinal tract (Freund et al., 2006; Liebscher et al., 2005) and restoration of serotonergic projections (Mullner et al., 2008) after spinal cord injury in several species, including macaque monkeys. Importantly, the potential of these antibodies to induce regeneration was decreased under complete spinal cord injury conditions, potentially due to the limited number of axons available to boost axonal sprouting and regeneration. This is in conjunction with our results which show no effect on bladder recovery of anti-Nogo-A effect in complete spinal cord injured rats (Figure 5 and 6). This leads to two hypotheses for the potential underlying mechanism of the Nogo-A-inhibition: first, spared descending fibers of the pontine micturition center are either building more and new synapses to the inhibiting

interneurons around the central channel below the lesion (more effectivity of spared fibers) or, second, that descending axotomized pontine micturition center neurons build new synapses above the lesion to propriospinal interneurons and thereby form a detour pathway around the lesion (newly built additional detour pathways), similar to the spontaneous regeneration mechanism found in neuron circuits from the gigantocellular reticular nucleus after cervical spinal cord injury (Bareyre et al., 2004; Filli et al., 2014). These promising findings pave the way for human trials with incomplete spinal cord injury patients suffering of neurogenic lower urinary tract dysfunction. A human phase one safety and tolerability trial with acute spinal cord injured patients was finished successfully in 2011 (NCT00406016). A Phase two randomized controlled multicenter trial is in preparation (http://cordis.europa.eu/project/rcn/198795_en.html). Given our promising results it would be of great interest to include urodynamic measurements in all patients for direct translation of our findings in to humans.

Main limitation of our study is that we investigated female rats only. We decided for this sex-specificity to avoid confounding by prostatic bladder outlet obstruction causing voiding dysfunction particularly in older male animals. In addition, male rats do have an increased mortality rate after spinal cord injury due to prostatic bladder outlet obstruction causing urinary tract infections upon severe pyelonephritis with urosepsis (practical experience of our lab). This is highly relevant in animal studies considering the 3Rs rule of Replacement, Reduction and Refinement (Flecknell, 2002; Kilkenny et al., 2010).

In conclusion, neurogenic lower urinary tract dysfunction due to incomplete spinal cord injury can be treated in a rat model with anti-Nogo-A antibody. Hence, anti-Nogo-A antibody is a potential causal treatment option for the life threatening neurogenic lower urinary tract dysfunction, in particular detrusor sphincter dyssynergia. In case of reproducibility of these findings in humans has anti-Nogo-A antibody a great potential to substantially change clinical practice and should that fore be used as soon as possible in a first human trial in spinal cord injury patients with incomplete lesions.

Furthermore, the urodynamic model and anti-Nogo-A antibody therapy should now be used to investigate the potential to treat other neurological disorders causing lower urinary tract dysfunction like stroke (Wahl et al., 2014) or multiple sclerosis (Hiroya Mizusawa et al., 2000).

5.6 References

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6 Concluding remarks and future directions

6.1 Conclusions

In chapter 3 we developed a novel urodynamic model that allows repetitive measurements of both, bladder and external urethral sphincter function at different time points in the same animal under fully awake conditions, this opens promising avenues to investigate the lower urinary tract in a translational approach. Our objective was in chapter 4 to investigate and compare bladder function in rats assessed by metabolic cage and by urodynamic measurements in fully awake animals. Bladder function of female Lewis rats was investigated in naïve animals by metabolic cage at baseline, 14-16 days after bladder catheter and external urethral sphincter electromyography electrode implantation in fully awake animals by urodynamics and again by metabolic cage. Lower urinary tract function assessed by metabolic cage or by urodynamic measurements in fully awake rats was indistinguishable. Thus, catheter implantation does not significantly change physiological bladder function. This shows, that urodynamic measurements in awake animals are an appropriate translational approach to study lower urinary tract function in health and disease, directly paralleling the human diagnostic procedures. In chapter 5 it was our objective to investigate if antibodies against the nerve fiber growth inhibitory CNS protein Nogo-A applied to the injured spinal cord could prevent the development of lower urinary tract dysfunction, in particular detrusor sphincter dyssynergia, following spinal cord injury. Our data indicate, that anti-Nogo-A-antibody treatment has beneficial effects on the lower urinary tract system, re-establishing a physiological status and preventing detrusor sphincter dyssynergia after incomplete spinal cord injury, presumably by influencing the neuronal wiring of descending micturition circuits. Anti-Nogo-A immunotherapy, which currently enters clinical trials for traumatic spinal cord injury, could therefore be a unique treatment option also for lower urinary tract dysfunction, which is a severe potentially life threatening condition in many diseases affecting the spinal cord.

6.2 Outlook

6.2.1 Underlying changes in the neuronal circuitry controlling the bladder function after spinal cord injury

Detrusor sphincter dyssynergia, the most dangerous type of neurogenic lower urinary tract dysfunction, appears two weeks after spinal cord injury in rats. Such a timeline could be well explained by sprouting and plasticity of interneurons settling around the central channel alone and or sprouting of sensory fibers from lamina two or three to deeper laminae, similar to findings observed in afferent fibers of quadriceps muscles after spinal cord injury (Krenz & Weaver, 1998). The detrusor sphincter dyssynergia develops both in incomplete and complete spinal cord injury to the same extend. This hints to a localization of the pathological processes below the lesion site. The very precise onset of the sphincter contraction exactly at the start of the voiding is clearly showing that any kind of afferent signaling must trigger the detrusor sphincter dyssynergia.

What is the underlying mechanism of the pathologic sphincter contraction during voiding?

Potential candidates are a sprouting of dorsal afferent fibers towards more ventral interneurons, forming new direct and stronger connections to sphincter activating interneurons or sprouting and strengthening of synaptic input from interneurons on the external urethral sphincter motoneurons. To address this question, it would be key to label the interneurons connected to the sphincter motoneurons. A perfect tool would be the monosynaptically restricted transsynaptic modified rabies virus. The genome of the virus is modified by a deletion of the rabies glycoprotein, which makes the virus not any more able to bud from the infected cells and spread transsynaptically (Wickersham et al., 2007). The missing rabies glycoprotein can be introduced in the neurons of interest by a separate virus, for example a modified lentivirus. This allows the modified rabies virus to spread transsynaptically but because the rabies glycoprotein is not present in the newly infected postsynaptic neuron, the virus cannot spread further. This

procedure results in a very robust and precise monosynaptic restricted transsynaptic tracing (Callaway, 2008). However, the mandatory intramuscular virus injection does currently not yet work in adult animals. But there seems light at the end of the tunnel: The Alvarez group from Atlanta, USA, claims that e.g. an approach with early injection of the helper virus (P15) allows for intramuscular modified rabies virus injections up to P60 with a robust expression in mice. As alternative approach has the same group 2016 at the Society for Neuroscience meeting a poster presented with preliminary results of successful injections in to transgenic adult mice (currently up to P45). They used RGT mice (Jax#024708) that express G protein and TVA receptor conditional to Cre recombination under the control of a CAG promotor inserted in to the Gt(ROSA)26Sor locus. Muscle expression was obtained by crossing of the RGT mice with ACTA1-Cre mice (Jax#006149), which drive Cre expression by the human alpha-skeletal actin (ACTA1) promotor. Injecting modified rabies virus intramuscular in these animals, they observed strong expression of the rabies virus in the muscle fibers and had much more transfected motoneurons in these animals, hence an increased percental transfection rate of the motoneurons in adult animals. In a second step, they have then obtained transgenic G protein expression in the motoneurons by crossing the RGT mice with Chat-IRES-Cre mice (Jax#006410), expressing Cre in cholinergic neurons without disrupting endogenous Chat expression. With these twice crossed animals, they were then able to have strong interneuron labeling, currently in up to P45 animals. This sounds like a very promising approach, however it would mean either to establish a similar urodynamic model in mice, what is clearly a challenge, or to produce similar transgenic rats with e.g. a CRISPER-Cas approach. If it becomes possible to label the interneurons connected to the sphincter motoneurons, immunostainings for different qualities of afferent fibers could be added and co-localized in naïve and spinal cord injured animals four weeks after injury, e.g. cGRP and Substance P for peptidergic C-fibers as well as IB4 for non peptidergic C-fibers. Additional the labeled interneurons should be further sub classified, e.g. for their main neurotransmitter using again different immunostainings.

An alternative approach also using transgenic animals would be to make a broad screening in a wide range of interneuron mouse lines, in particular interneuron subgroups that are known to be positioned around the central channel (Bikoff et al., 2016; Jessell, 2000; Zagoraiou et al., 2009). Lines for afferent neurons could be included in a similar approach. A quick and broad screening for several transgenic mouse lines could be made using a metabolic cage setup. Measurements should be performed in conditional animals before and after silencing. Suspicious mouse lines with e.g. reduced void volumes, prolonged voiding durations or other abnormalities after silencing should then be further investigated using a full urodynamic assessment including external urethral sphincter EMG. In case of positive findings in urodynamic measurements the next step would be to combine the mouse lines with spinal cord injuries and again baseline with detrusor sphincter dyssynergia followed by silencing and potentially modified micturition afterwards. Identification of the potentially underlying sub groups of detrusor sphincter dyssynergia would be of great value for both the understanding of the mechanism and the development of new treatment approaches.

6.2.2 What are the underlying mechanisms of the anti-Nogo-A antibody therapy on bladder function?

We observed a strong treatment effect of anti-Nogo-A antibody therapy on detrusor sphincter dyssynergia following incomplete spinal cord injury. Here it most likely prevents early detrusor sphincter dyssynergia developing altogether. No treatment effect was observed in complete spinal cord injured animals. However, we do not yet understand the specific mechanism of action of the anti-Nogo-A antibody treatment.

What are the underlying mechanisms of anti-Nogo-A antibody treatment following spinal cord injury to prevent the development of detrusor sphincter dyssynergia?

There are two potential mechanisms that are likely, one is that the anti-Nogo-A antibody causes plastic changes below the lesion, in particular in the interneurons around the motoneurons of the external urethral sphincter, e.g. inhibiting pathways could be strengthened. To show such a mechanism a similar approach, as described above, with a modified rabies virus could be used in combination with immunohistochemical staining's like vGLUT and vGAT2 to quantify the synaptic input of the labeled interneurons on the sphincter motoneurons, e.g. to quantify the number of both inhibiting and activating synapses per 100µm cell surface. However, there is a second more likely mechanism of action that may cause the treatment effect. The fact that we do not observe a treatment effect in complete transected animals hints towards the importance of persisting or newly built descending or ascending pathways of the bladder across the lesion, i.e. from the pontine micturition center to the spinal micturition centers and back. There are several potential explanations in line with this hypothesis. First it might be that the persisting fibers are strengthened under treatment and become plastic below the lesion and form more functional synapses below the lesion. Second, axotomized fibers could grow newly through the lesion and form new functional synapses below the lesion. Third, axotomized fibers form more synapses above the lesion on propriospinal interneurons, which form a detour pathway around the lesion with functional connections to the spinal micturition centers below the lesion, similar process are observed in the reticulospinal fibers after lesions (Filli et al., 2014).

Or it might be that all three contribute towards better bladder function after spinal cord injury under anti-Nogo-A treatment. A first step to assess the density of descending fibers of the pontine micturition center by a immunohistochemically staining for corticotropin releasing factor above and below the lesion, as well as in the spinal target regions at sacral level 2, to perform a densitometry analysis around the central channel. Corticotropin releasing factor is reported to be highly specific for the pontine micturition center fibers in the spinal cord and could hence be used for its quantification (Klausner & Steers, 2004; Nemeroff et al., 1984). In a second step, the further strategy would be defined by the results from step one. If the effect is more expected above the lesion by increased number of synapses to propriospinal interneurons, a combined retrograde tracing and staining approach with conventional dextran tracer injections in to the spinal micturition centers and anti corticotropin releasing factor staining for a quantitative analysis of the number of synaptic connections above the lesion could be promising. Altenatively, if the results from phase one point towards mechanisms below the lesion, a combined approach with retrograde trans synaptic tracing with modified rabies virus to identify the interneurons and anti-corticotropin releasing factor staining could help shine a light on this question.

6.2.3 Anti-Nogo-A antibody therapy for spinal cord injury in the chronic phase

Anti-Nogo-A antibody has a potent treatment effect if applied in the very acute phase after injury. However, most spinal cord injury patients are in the chronic phase after injury and would not qualify for this likely causal therapy.

Is it possible to treat neurogenic lower urinary tract dysfunction, in particular detrusor sphincter dyssynergia, in the chronic phase after injury with anti-Nogo-A antibody therapy?

It is likely that the very different milieu of apoptotic, inflammatory and growth factors is important for an effective treatment with the growth promoting anti-Nogo-A antibody. Hence it is not clear if anti-Nogo-A antibody therapy alone would substantially change the bladder function in the chronic situation after spinal cord injury. Besides enabling the growth, any kind of stimulation is likely to be needed to push the neuronal circuitry again in to a plastic stage, i.e. any kind of bladder rehabilitation training. Classical approaches in spinal cord injured patients seem difficult from two different points of view. First, many of them have lost the bladder sensation, thus the full bladder cannot be sensed, a crucial part of rehabilitation. Second, the bladder contraction is a parasympathetic process and can therefore not like somatic muscles be contracted at direct will. Bladder contraction is more like a passive process that can be supported but not directly initiated. It seems very difficult to create a successful bladder rehabilitation paradigm without any technical device, in particular without sensation. Therefore the circuitry e.g. may be stimulated and activated by deep brain stimulation of the pontine micturition center. A comparable approach was just recently successfully published for locomotion improvement under deep brain stimulation of the midbrain locomotor region (Bachmann et al., 2013). The activation of the spared descending fibers by supra threshold deep brain stimulation could likely have already a direct treatment effect by strengthening of these fibers, but the other interesting hypothesis would be that a strong physiologic activation of the bladder circuitry, i.e. a rehabilitation training could be used in combination with anti-Nogo-A antibody therapy to treat

detrusor sphincter dyssynergia in chronic spinal cord injured patients. Other less invasive stimulation devices like pelvic floor stimulation or sacral nerve modulation could be alternatives. However deep brain stimulation of the pontine micturition center is likely to be the ideal target for rehabilitation stimulation.

6.2.4 Anti-Nogo-A antibody therapy in human patients

We observed strong treatment effects of the anit-Nogo-A antibody for the life-threatening detrusor sphincter dyssynergia in incomplete spinal cord injured animals, thus it is possible for this to be the first causal treatment option for neurogenic lower urinary tract dysfunction. Given the fact that in 2011 a phase one safety clinical trial was successfully finished with anti-Nogo-A antibody in human spinal cord injury patients and with our new results; it is of great importance to include in the comparative phase two trial, that is currently in preparation, a detailed bladder function assessment. It would be of great importance to include a urodynamic assessment including external urethral sphincter EMG in the acute phase and six months after injury. In case of a successful phase two and a good safety profile further trials should include patients suffering of bladder dysfunction due to Multiple Sclerosis, Spina bifida, Stroke and others. Another follow up trial should include the treatment in chronic spinal cord injury patients, ideally in combination with bladder rehabilitation, but there are further animal trials needed to first identify the ideal paradigm.

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7 Appendix

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Publications

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